MODIFICATION OF ADENOVIRUS CAPSID PROTEINS FOR GENE THERAPY APPLICATIONS

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ABSTRACT

Adenovirus (Ad) is the most commonly used viral vector in gene therapy applications to date for a broad range of diseases. Although Ad-based viral vectors have many advantages in a variety of gene therapy designs, the commonly used adenoviral vectors have several key shortcomings. Those shortcomings include (1) inefficient transduction in cell types devoid of Ad’s native receptors and the incapability of gene delivery to targets behind physical barriers; (2) restricted accessibility to the central nerve system due to the existence of the blood-brain barrier (BBB); (3) lack of a useful strategy and platform to generate multi-functionality displaying Ad vectors as to achieve functional integration or synergism, which could improve Ad’s utility. We hypothesized that 1) incorporation of targeting ligands onto fiber protein would broaden the delivery range of Ad vectors in terms of cell types; 2) retargeting adenoviral vectors to the native transcytosis pathway in BBB endothelial cells would allow efficient gene delivery into the brain; 3) incorporation of multiple heterologous peptide ligands into a single Ad virion at the minor capsid protein IX (pIX) locates could allow for the display of multiple functionalities simultaneously, thus giving rise to functional integration or synergistic effect. To achieve the modification of Ad fiber and consequent expansion of gene delivery targets, the protein transduction domain of HIV-1 Tat protein (PTDtat) or melanotransferrin protein (MTf) was incorporated into the fiber knob protein via a genetic or non-genetic approach, respectively. The derived Ad vector incorporated with
PTDtat showed expanded tropism and enhanced transduction efficiency in various tumor cells; the derived Ad vector incorporated with MTf achieved gene delivery across the BBB in an *in vitro* model. To incorporate three heterologous peptide ligands into a single Ad virion at the pIX locale, three modified pIX genes were genetically engineered and inserted into the Ad genome, resulting in the expression and incorporation of three different types of IX-ligand fusion proteins. The results indicated that capsid modification is a potent and useful strategy to enhance the efficacy of Ad vectors in gene therapy applications. However, this study mainly focused on the principle of capsid-modifying strategies, and further optimizations are necessary for their application for clinical trials.
DEDICATION

To my families, my teachers, and my friends: it’s your constant help, support, and encouragement that strengthen me and make this accomplishment possible.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>The Status of Gene Therapy</td>
<td>1</td>
</tr>
<tr>
<td>The Status of Cancer Gene Therapy</td>
<td>2</td>
</tr>
<tr>
<td>The Status of Gene Therapy for Ocular Disorders</td>
<td>5</td>
</tr>
<tr>
<td>Transcytosis for Therapeutic Delivery into the Central Nervous System</td>
<td>10</td>
</tr>
<tr>
<td>Viral Vectors for Gene Therapy</td>
<td>15</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>18</td>
</tr>
<tr>
<td>Capsid-modified Adenovirus for Gene Therapy</td>
<td>25</td>
</tr>
<tr>
<td>Capsid Protein Fiber: A Potential Locale for Re-targeting Adenovirus</td>
<td>28</td>
</tr>
<tr>
<td>Protein Transduction Domain (PTD) of HIV-1 Tat</td>
<td>31</td>
</tr>
<tr>
<td>Capsid Protein IX: A Potential Locale to Carry Multifunctionalities</td>
<td>33</td>
</tr>
<tr>
<td>GENETIC INCORPORATION OF PROTEIN TRANSDUCTION DOMAIN OF Tat INTO ADENOVIRUS TYPE 5 FIBER</td>
<td>39</td>
</tr>
<tr>
<td>DIRECTING ADENOVIRUS ACROSS BLOOD-BRAIN BARRIER VIA MELANOTRANSFERRIN TRANSCYTOSIS PATHWAY</td>
<td>67</td>
</tr>
<tr>
<td>DERIVATION OF A TRIPLE MOSAIC ADENOVIRUS BASED ON MODIFICATION OF THE MINOR CAPSID PROTEIN IX</td>
<td>98</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>135</td>
</tr>
<tr>
<td>Targeting Adenoviral Vectors to Brain Tumors</td>
<td>135</td>
</tr>
<tr>
<td>Redirecting Adenoviral Vectors Across the BBB</td>
<td>143</td>
</tr>
<tr>
<td>A Powerful Multifunctional Adenoviral Platform for Cancer Therapy</td>
<td>150</td>
</tr>
<tr>
<td>LIST OF REFERENCES</td>
<td>159</td>
</tr>
<tr>
<td>--------------------</td>
<td>-----</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>193</td>
</tr>
<tr>
<td>A INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE ANIMAL PROTOCOL APPROVAL</td>
<td>194</td>
</tr>
<tr>
<td>B INSTITUTIONAL REVIEW BOARD FOR HUMAN USE DESIGNATION OF NOT HUMAN SUBJECTS RESEARCH</td>
<td>196</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table                                                                 page

DIRECTING ADENOVIRUS ACROSS BLOOD-BRAIN BARRIER VIA MELANOTRANSFERRIN TRANSCYTOSIS PATHWAY

1  A representative set of TEER data before and after transcytosis assay.....................77
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A stylized section of Adenovirus type 5 particle based on the current understanding of its protein and DNA components</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Transcription of the adenovirus genome</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>The pathway of Ad5 infection</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>GENETIC INCORPORATION OF PROTEIN TRANSDUCTION DOMAIN OF TAT INTO ADENOVIRUS TYPE 5 FIBER</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Diagram of PTDtat modified Ad5 vector</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>Ad5.PTDtat showed similar CAR-binding activity to unmodified Ad5 vector in an ELISA-based binding assay</td>
<td>47</td>
</tr>
<tr>
<td>3</td>
<td>PTDtat modification promoted Ad5 binding to cell surfaces</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td>Ad5.PTDtat exhibited enhanced gene transfer efficacy in a variety of tumor cells</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>Competitive inhibition assay showing the enhanced gene transfer efficacy of Ad5.PTDtat was mediated by the PTDtat motif</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>PTDtat modification of Ad5 fiber enhanced in vivo gene delivery efficacy of the vector</td>
<td>54</td>
</tr>
<tr>
<td>1</td>
<td>DIRECTING ADENOVIRUS ACROSS BLOOD-BRAIN BARRIER VIA MELANOTRANSFERRIN TRANSCYTOSIS PATHWAY</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Generation of bi-specific adaptor protein sCAR-MTf</td>
<td>73</td>
</tr>
<tr>
<td>2</td>
<td>sCAR-MTf mediated Ad5 transcytosis in the in vitro BBB model</td>
<td>75</td>
</tr>
<tr>
<td>3</td>
<td>Infectivity of the transcytosed Ad5 viral particles</td>
<td>78</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Characterization of the temperature- and dose-dependence of sCAR-MTf mediated Ad5 transcytosis</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>Directionality of sCAR-MTf mediated Ad5 transcytosis</td>
<td>81</td>
</tr>
</tbody>
</table>

### DERIVATION OF A TRIPLE MOSAIC ADENOVIRUS BASED ON MODIFICATION OF THE MINOR CAPSID PROTEIN IX

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schema of Ad pIX modification</td>
<td>103</td>
</tr>
<tr>
<td>2</td>
<td>Western blotting analysis of Ad vector containing triple pIX modifications</td>
<td>105</td>
</tr>
<tr>
<td>3</td>
<td>Direct visualization of pIX-modified Ad vector by fluorescence microscopy</td>
<td>106</td>
</tr>
<tr>
<td>4</td>
<td>Presentation of modified IX proteins on Ad capsid surface</td>
<td>108</td>
</tr>
<tr>
<td>5</td>
<td>Cell binding and infectivity of pIX-modified Ads</td>
<td>109</td>
</tr>
<tr>
<td>6</td>
<td>Thermostability and growth kinetics of pIX modified Ads</td>
<td>112</td>
</tr>
<tr>
<td>7</td>
<td>Immuno-gold electron Microscopy on pIX-modified Ads</td>
<td>114</td>
</tr>
<tr>
<td>8</td>
<td>Generation of pIX mosaic Ad5 by co-infection</td>
<td>115</td>
</tr>
</tbody>
</table>

### DISCUSSION

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Effect of filipin on transcytosis in the in vitro BBB model</td>
<td>148</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>Ad5</td>
<td>human adenovirus serotype 5</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
<td></td>
</tr>
<tr>
<td>CAR</td>
<td>coxsackievirus B and adenovirus receptor</td>
<td></td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
<td></td>
</tr>
<tr>
<td>HSV-TK</td>
<td>herpes simplex virus type 1 thymidine kinase</td>
<td></td>
</tr>
<tr>
<td>MLP</td>
<td>adenovirus major later promoter</td>
<td></td>
</tr>
<tr>
<td>mRFP1</td>
<td>monomeric red fluorescent protein</td>
<td></td>
</tr>
<tr>
<td>MTf</td>
<td>melanotransferrin protein</td>
<td></td>
</tr>
<tr>
<td>PFU</td>
<td>plague-forming unit</td>
<td></td>
</tr>
<tr>
<td>pIX</td>
<td>adenovirus protein IX</td>
<td></td>
</tr>
<tr>
<td>RGD</td>
<td>extracellular matrix tripeptide arginine-glycine-aspartate motif</td>
<td></td>
</tr>
<tr>
<td>sCAR</td>
<td>soluble extracellular domain of CAR</td>
<td></td>
</tr>
<tr>
<td>sCAR-MTf</td>
<td>soluble CAR-melanotransferrin fusion protein</td>
<td></td>
</tr>
<tr>
<td>TCID50</td>
<td>tissue culture infectious dose 50</td>
<td></td>
</tr>
<tr>
<td>TEER</td>
<td>transendothelial electrical resistance</td>
<td></td>
</tr>
<tr>
<td>VP</td>
<td>viral particle</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION

The Status of Gene Therapy

Gene Therapy represents a new paradigm in the treatment of human diseases by the insertion of therapeutic genes into individuals’ cells. The originally conceived rationale of gene therapy for a certain disease is based on our knowledge that the disease is caused by the malfunction of specific genes and that the pathophysiological phenotypes of the disease can be rescued by effectively compensating the affected cells with normal, functional counterparts. This rationale can be applied to many known inherited disorders such as numerous genetic metabolic diseases and a variety of inherited immune deficiency disorders. Along with recent and remarkable progress in our understanding of the molecular biology and the mechanisms of many acquired diseases, such as cancers, neurodegenerative disorders, and infectious diseases, the concept of gene therapy has been extended from solely “functionally compensating for a defective gene” to “imparting a new function in order to induce a putative therapeutic function”, which has been investigated in many experimental studies and clinical trials (see the references and overviews). The practical usage of gene therapy is currently classified into two cellular categories: somatic stem cells, e.g. hematopoietic stem cells, and terminally differentiated, postmitotic cells, e.g. hepatocytes or neurons. Gene therapy is still in its infancy and underwent a severe setback when a male patient suffering from ornithine transcarbamylase (OTC) deficiency died during the participation in a clinical trial in 1999. However, encouraging results of gene therapy are starting to emerge and it has
been demonstrated as a potential alternative or novel strategy for many diseases. Since the first gene therapy clinical trial to treat metastatic melanoma in 1989\textsuperscript{14} and first therapeutic trial to treat a form of severe combined immune deficiency in 1990\textsuperscript{15}, more than 1300 clinical trials have been performed or are being performed to date\textsuperscript{12}.

**The Status of Cancer Gene Therapy**

Thus far, the gene therapy clinical trials have been predominantly aimed at the treatment of cancer\textsuperscript{12}. Cancer remains the second leading cause of death worldwide, and caused approximately 7.9 million deaths (13\% of all deaths) in 2007 according to the report from the World Health Organization (WHO, “The top 10 causes of death”). Cancer affects populations of all ages and races, and diminishes the quality of life as well as causes long-term economical burden\textsuperscript{16-18}. Among all treatment methods for cancer, gene therapy represents a novel molecular measure that has the potential of anti-tumor efficacy with selectivity and safety\textsuperscript{19}, supported by the fact that 65.2\% of all gene therapy clinical trials to date are targeted at cancer\textsuperscript{12}. Extreme focus in cancer gene therapy has been placed on the development of novel cancer gene therapy strategies with distinct underlying biological mechanisms including, immunotherapy\textsuperscript{20}, tumor suppressor gene therapy\textsuperscript{21}, suicide gene therapy\textsuperscript{22}, oncolytic viral therapy\textsuperscript{9}, and combination of gene therapy with chemotherapy or radiotherapy\textsuperscript{23-25}.

Immunotherapy against cancer provides therapeutic or preventive anti-tumor responses through modulating the host immune system or reversing the immunosuppressive microenvironment of cancer\textsuperscript{26, 27}. Means of immunotherapy include local manipulation of proinflammatory and immunosuppressive cytokines and genes\textsuperscript{28},
complementation of class I major histocompatibility complex\textsuperscript{29, 30}, and the activation of tumor-specific T lymphocytes through genetic manipulation of antigen-presenting cells\textsuperscript{31}.

The ability to evade apoptosis is a major hallmark of most or perhaps all types of cancer\textsuperscript{32}. The induction of apoptosis involves a number of tumor suppressor genes, which mediate cellular checkpoint control and hence prevent the further passage of cellular damage. For example, p53 tumor suppressor gene is critical in regulating apoptotic signaling and determining the cell fate, and its function is turned off in about half of human tumors\textsuperscript{33, 34}. Therefore, restoration of p53 function has been extensively studied in cancer gene therapy and was shown to have an anti-tumor effect due to p53-induced apoptosis in tumor cells\textsuperscript{35, 36}.

A suicide gene, such as herpes simplex virus-1 thymidine kinase (HSV-TK)\textsuperscript{37} or \textit{Escheria coli} cytosine deaminase\textsuperscript{38}, encodes an enzyme capable of converting a prodrug into a cytotoxic metabolite which induces cell death. Administration of the suicide gene into tumor cells followed by prodrugs has been extensively studied and suggested as a potential alternative to conventional chemotherapy. Compared to conventional chemotherapy, suicide gene therapy or molecular chemotherapy\textsuperscript{39, 40} can be restricted to tumor cells by targeting the vector to tumor specific antigens (e.g. carcinoembryonic antigen\textsuperscript{41}) or a unique transcriptional status (e.g. expression driven by tumor-specific promoter\textsuperscript{42, 43}), thus avoiding toxicity to normal cells, supported by several clinical trials of breast cancer\textsuperscript{44} and prostate cancer\textsuperscript{45}.

Several types of virus have shown cytotoxic and cell lytic capability while replicating, such as adenovirus (Ad), herpes simplex virus (HSV), vaccinia and reovirus, etc. that have been utilized as tumor cell-killing agents and tested in a number of clinical
trials (see the review\textsuperscript{9}). The key to virotherapy relies on the viral replication and spread in tumor cells with high selectivity, which theoretically requires only a small amount for initial infection and yields a tumor-wide effect. To reach high selectivity of viral replication, much effort has been focused on the development of conditionally replicative viruses as well as the characterization of related immune responses and safety issues. For example, a modified picornavirus (called Seneca Valley Virus, SVV-001) has been developed and was shown to selectively kill retinoblastoma in a murine model\textsuperscript{46}.

These numerous cancer gene therapy strategies provide a wide range of new techniques for fighting cancer and powerful alternatives to conventional cancer therapy. Despite recent advances in the preliminary proof-of-principle and preclinical studies of these strategies, one of the major obstacles for all cancer gene therapy protocols remains to be overcome, i.e. low transduction efficiency of the therapeutics and the inability to transduce the entire tumor cell population.

Adenovirus is one of the most commonly used viral vectors in cancer gene therapy. In addition to Ad’s traditional application in oncolytic viral therapy based on Ad’s native characteristic of cell lysis, Ad has also been widely used in other major cancer gene therapy strategies. Several advances in Ad vector development appear to be able to resolve the major obstacle of cancer gene therapy that was mentioned earlier. First, modification of the Ad capsid with targeting ligands directed against tumor antigen boosted Ad transduction in tumor cells \textit{in vitro} and \textit{in vivo}\textsuperscript{47, 48}. Second, Ad capsid labeled with fluorescent proteins allowed for dynamic and direct monitoring of the physical location and replication of viral particles \textit{in vivo}\textsuperscript{49-51}, which greatly facilitated oncolytic viral therapies. Third, the genetic fusion of suicide proteins on Ad capsid
The Status of Gene Therapy for Ocular Disorders

With the tremendous progress that has been made in the last two decades in dissecting the molecular bases of a variety of ocular disorders, it has become feasible to treat these diseases with gene therapy at the molecular level. The rationales underlying ocular gene therapy strategy are: gene replacement therapy is feasible to reverse inherited retinal degenerations due to loss-of-function mutations in critical genes in retinal pigment epithelial (RPE) cells and other retinal cells (mainly photoreceptors); ribozyme or small interfering RNA can be used to suppress gene over-expression owing to gain-of-function mutations and thus ameliorate mutation-induced retinal degenerations; gene transfer of
therapeutic proteins, such as neurotrophic or immune suppressive factors, can be used to slow down the progression of, or can be used to assist the treatment of, acquired ocular diseases. The human eye is a superior treatment target of gene therapy for several reasons: highly developed techniques are available, such as fundus imaging, electrophysiological and behavioral test, which precisely and readily examine the phenotype of disease and therapeutic effects \textit{in vivo}; a large number of inherited and acquired ocular disorders have been identified in molecular level (e.g. Retinitis Pigmentosa\textsuperscript{55}); the human eye has a highly compartmentalised anatomy and a unique immune environment, which makes the experimental design much easier; a number of appropriate animal models are available and have been rigorously evaluated in the laboratory in the preparation for the development of clinical therapies\textsuperscript{56}.

Gene therapy strategy has been explored in many ocular disease scenarios: from inherited to acquired disorders, from ocular anterior and peripheral segments to ocular fundus, and from corneal epithelial cells to retina neuronal photoreceptors (see the review\textsuperscript{57}). Even though gene therapy has not been demonstrated to cure most of these diseases in humans, it seems promising since the proof-of-principle of treating many ocular diseases has been established in the animal models for a number of diseases, including proliferative retinopathies and severe genetic ocular diseases\textsuperscript{58-70}. It is noteworthy and intriguing to every gene and ocular therapist that the first demonstration of gene therapy efficacy in the human has emerged recently in a form of inherited retinal degeneration disease, called Leber’s congenital amaurosis (LCA)\textsuperscript{71,72}. LCA is a severe blindness-causing inherited disease affecting approximately 1 in 81,000 of the population with an onset as early as at the first few months of life\textsuperscript{73}. LCA is inherited in an
autosomal recessive pattern with mutations found in 9 genes, which are predominantly expressed in retina photoreceptors or RPE cells. Maguire et al. and Bainbridge et al. focused on one particular form of LCA caused by null mutations in RPE65 gene which encodes the isomerohydrolase in visual cycle\textsuperscript{74} converting \textit{all-trans} retinyl esters to \textit{11-cis} retinoids in RPE. The deficiency of RPE65 protein results in lack of rhodopsin pigment and accumulation of all-trans retinyl ester and its precursors in RPE and photoreceptor cells, and is correlated with pathologic cell phenotypes and retinal dystrophy and degeneration\textsuperscript{75-77}. Both groups showed that exogenously expressed RPE65 proteins partially rescued visual function of some patients and suggested that LCA2 may be subject to gene-replacement therapy.

Both viral and nonviral vectors have been tested in a number of ocular gene therapy clinical trials. Viral vectors have been predominantly used thus far due to their high gene transfer efficiency in various cell types (dividing and non-dividing cells) and long duration of gene expression. Particularly in ocular gene therapy, adenoviral-mediated gene transfer has been extensively and widely used in most compartments in the eye including corneal endothelial and epithelial cells, lens, iris, trabecular meshwork, retinal and subretinal areas (see the reviews\textsuperscript{57, 78, 79}). In this regard, gene therapy has been used to treat corresponding ocular disorders or exert positive effects to assist other therapies. Adenoviral vectors carrying suicide genes have also been directly administrated into ocular tumor cells of retinoblastoma, which is the most common primary ocular malignancy of childhood\textsuperscript{80, 81}. However, adenoviral vector-mediated gene transfer in human eyes or animal models is usually associated with short-term gene expression and high inflammatory responses, which will be discussed later on. Adeno-
associated virus (AAV)-based vectors are predominantly used to transfect cells in retinal and subretinal areas, including photoreceptors, ganglion cells, Müller cells and RPE cells, in order to treat retinitis pigmentosa, glaucoma, neovascular diseases, and some optic nerve diseases. AAV-mediated gene expression can be sustained for several years without causing severe adverse effects, probably due to AAV’s small size and the lack of pathogenicity of recombinant AAV, which is devoid of all viral coding sequences. Lentivirus is another common viral vector in ocular gene therapy, which integrates its genome into host genome and achieves long-term gene expression. Lentiviral vectors have been investigated in transducing corneal endothelium, trabecular meshwork, RPE, and photoreceptors to treat corneal haze, glaucoma, and retinal degenerative diseases.

Human eyes are considered as immune privileged due to the existence of several physical barriers between the eyes and blood circulation, such as the blood-brain barrier (BBB) and the blood-retina barrier (BRB), as well as the secretion of several immunoreactive molecules. However, immune responses are nevertheless triggered by the delivered viral vectors and the embedded transgenes. Due to the existence of BBB and/or BRB, almost all ocular gene therapy protocols administrated the vectors via direct injection into the eyes intravitreally, intracameraly, or subretinally for particular cell types, which induces local inflammatory responses. For treatment of severe ocular diseases, mild and transient inflammatory responses due to necessary substances and procedures are acceptable. However, one must be aware that the caused inflammatory and other immune responses likely induce long-term or even permanently damage to visual function when the terminally differentiated cell types in retina are targeted. These cells, especially photoreceptors and ganglion cells which have delicate synaptic
connections with many other cell types, have already exited cell cycle and are likely irreplaceable. The development and differentiation of these cells are lengthy and require a perfect timing along with the development of neighboring cells\textsuperscript{96, 87}, thus it is very hard to replenish these cell types. Therefore, inflammatory and other immune responses and their possible outcomes should be carefully considered in the development of any ocular gene therapy protocols.

Following ocular administration of therapeutic vectors, cell-mediated immune responses can limit the transgene expression, and the limitation effects may differ between anterior and posterior segments in the ocular, with a more severe inflammatory effect after intravitreous infection\textsuperscript{88}. Antigen-presenting cells play important roles in cell-mediated immune responses, which probably are RPE cells and some dendritic cells in choroid and retinal microglia\textsuperscript{89, 90}. Since adenovirus but not AAV transduces RPE cells, cell-mediated immune responses are usually observed in adenovirus-mediated gene transfer but not in AAV-mediated gene transfer\textsuperscript{90}. However, humoral immune responses, mainly antibody responses, were indeed elicited against AAV antigen and AAV-mediated transgene products\textsuperscript{58, 91, 92}, even though these humoral responses against AAV did not seem to affect the re-administration of such vectors to achieve high gene expression\textsuperscript{93}. Moreover, high dose administration of recombinant AAV vector may induce insertional mutation in host cells due to AAV genome integration, which is likely associated with an increased incidence of hepatocellular carcinoma in neonatal mice\textsuperscript{94, 95} and other malignancies (http://www4.od.nih.gov/oba/rac/Transcript3-7-011.pdf).
Transcytosis for Therapeutic Delivery into the Central Nervous System

The mammalian central nervous system (CNS) is the most complicated and sophisticated organ that is highly specialized and localized for its unique function and its massive magnitude of networking among cellular components. The high specialization and differentiation also greatly limits its capacity of regeneration and self-repair. CNS diseases, which are usually characterized by permanent and disabling neuronal loss, remain one of the leading causes of human death and disability in the world, and account for most hospitalizations and prolonged care in all categories of human disease.

A tremendous number of advances have been made in the last two decades in the understanding and treatment of numerous types of CNS diseases, such as brain tumors, Parkinson’s disease, Alzheimer’s disease, spinal cord injury as well as several new targets of CNS therapy, including epilepsies and Arteriovenous malformations (AVMs)\(^96\). For example, by considering the relentless and gradual progression from cellular dysfunction to death in many neurodegenerative diseases, the diseases at early stage might be rescued to some extent by neuroprotective strategies. Gene therapy has been utilized to treat neurodegenerative diseases through the delivery of neurotrophic factors, anti-oxidant, and/or anti-apoptotic molecules locally\(^97\). Cell therapy also shows promise for metabolic, genetic neurodegenerative diseases, CNS lesion, and even glioma tumors through the engraftment of neural progenitor cells\(^98, 99\) or the systemic infusion of genetically manipulated neural stem cells\(^100, 101\).

Patients are still suffering from many CNS diseases, including ocular diseases, due to the low efficiency in the medical treatment to those delicate and complicated tissues/organs in the CNS. A major bottleneck in treating CNS diseases is the lack of an
efficient drug delivery method. Currently, the majority of gene therapy protocols utilize direct intracranial injection to deliver therapeutic compounds into CNS parenchyma or ventricular regions (vitreous body or subretinal region for ocular gene therapy), which potentially cause physical injuries, local inflammatory responses, and can only mediate transgene expression in a small area around the injection sites.

The progress in improving the methodology of drug delivery into the CNS greatly lags compared with advances in the development of delivering therapeutics and devices in other organs, which is largely owing to the existence of several physical barriers between the blood circulation and CNS organs, such as blood-brain barrier (BBB). BBB is a unique membrane and acts as a dynamic filtering system between the brain and the blood stream. On one hand, BBB segregates the brain from the blood stream since the composition of blood and its temporal variation due to stress, food uptake/starvation would hamper the molecular microenvironment of neuron. On the other hand, BBB mediates substance exchange through numerous types of importing and exporting mechanisms to meet the high nutritional requirement of the brain\textsuperscript{102}. Therefore, BBB not only plays the role as a “barrier” but also plays the role as a “carrier”, responsible for regulating the influx and efflux of essential substances and for mediating the metabolic homeostasis and ion microenvironment\textsuperscript{103}. Furthermore, the transportation across the BBB is highly selective for molecules. For example, the BBB prevents the penetration of many small molecules (e.g. histamine and other immune regulatory molecules and neutotoxic metabolites) through a number of efflux pumps (e.g. p-glycoprotein and its families) expressed on the luminal side of vasculature\textsuperscript{104,105}. The BBB also prevents the passage of most large molecules and cells (e.g. pathogenic proteins, virus, and peripheral
lymphocytes) through high resistance tight junctions on vascular endothelium. Therefore the BBB blocks the uptake of more than 98% of all potential neurotherapeutics\textsuperscript{106}.

Four types of cell are usually found in the conformation of BBB, which are endothelial cells, astrocytes, pericytes, and neurons\textsuperscript{107}. The permeability and high resistance of BBB is believed to be determined by endothelial cells, which lack fenestrations and endocytic vesicles and are sealed with high resistant tight junctions as demonstrated by electron microscopy\textsuperscript{108}. The BBB has different physiological strategies to transport different molecules according to their unique properties. Small molecules that have less than 400-600 Dalton in molecular weight and forms less than 8-10 hydrogen bonds with water can passively diffuse across the BBB in a lipid-mediated manner\textsuperscript{109}. Large lipid-soluble molecules and water-soluble molecules are transported across the BBB by means of carrier-mediated transport (CMT), active-efflux transport (AET), or receptor-mediated transcytosis (RMT)\textsuperscript{107}. CMT is usually used to bidirectionally transport water-soluble small molecules across the BBB, including glucose, amino acids, nucleosides, thyroid hormone, and water-soluble vitamins. AET catalyses unidirectional efflux of many small metabolic molecules from the abluminal side (brain) to the luminal side (blood) through numerous efflux pumps, e.g. p-glycoprotein. Many therapeutic molecules are AET substrates, and as a result most drugs can not reach significant accumulation in the brain by systemic delivery. RMT system mediates either bidirectional or unidirectional transport of relatively large molecules through protein-specific receptors on the luminal and abluminal membrane of BBB endothelial cells, therefore RMT system is considered highly specific. The targets of the RMT system are endogenous circulating peptides and proteins such as insulin and transferrin via the
insulin and transferrin receptors\textsuperscript{110, 111}, or low-density lipoprotein (LDL) by LDL receptors\textsuperscript{112}. The process of bidirectional RMT includes initial specific recognition of the ligand to its cellular receptors on the BBB endothelial cells, endocytosis of the ligand-receptor complex, intracellular trafficking, and exocytosis with the release of the ligand.

To circumvent the BBB for large therapeutic molecules across it, many strategies have been investigated. Traditional invasive methods include intracerebroventricular infusion or intracerebral implants\textsuperscript{113, 114} and temporary disruption of the BBB endothelium by chemical or physical means such as hypertonic solution mannitol and low-frequency ultrasound (see the reviews\textsuperscript{115, 116}). Albeit showing promise in delivery efficiency, the risks of infection and neuropathological abnormality make many of these strategies unacceptable for clinical applications.

As a non-invasive strategy compared to invasive methods, a variety of receptor-mediated transcytosis pathways have been evaluated to overcome the BBB for drug delivery into brain. To hijack RMT pathways for delivering therapeutic molecules, fusion protein can be constructed in which the drug molecule is linked to the antibody against the RMT receptor or directly to the endogenous ligand of the receptor. For example, brain-derived neurotrophic factor (BDNF) was conjugated with a MAb against transferrin receptor (TfR), so that TfRs would carry the intravenously administrated BNDF-MAb conjugates across the BBB. Even though the efficacy of this strategy has been consistently demonstrated in a variety of animal models including ischemia stroke and neurodegenerative diseases such as Alzheimer’s diseases and Parkinson disease\textsuperscript{117-121}, the overall efficiency of the fusion protein (drug-MAb) transcytosis across the BBB is very
low (0.2–0.4% of intravenously injected dose\textsuperscript{118, 122}) which is a major limitation for clinical usage.

Other than TfR-mediated RMT, many more endogenous transport pathways are being investigated to find better candidates for drug delivery across the BBB with higher efficiency, such as melanotransferrin (MTf, also known as p97)\textsuperscript{123}, and receptor-associated protein (RAP)\textsuperscript{124}. The glycosylated MTf is a transferrin homolog originally identified in human melanoma\textsuperscript{125, 126}. MTf exists in a membrane bound form with a glycosylphosphatidylinositol (GPI) anchor as well as a soluble form, and plays important roles in many physiology processes (see the review\textsuperscript{127}). For example, MTf is considered to contribute to iron transportation into the brain\textsuperscript{128}, although its role may not be essential since the MTf knockout mice showed no difference in iron metabolism\textsuperscript{129}. MTf is also considered as a biochemical marker of Alzheimer’s disease (the soluble form\textsuperscript{130}) and a hallmark of tumor proliferation and metastasis with cell surface plasminogen activation involved\textsuperscript{131, 132}. The membrane bound MTf is composed of 719 amino acid residues including two homologous extracellular domains of 342 and 352 amino acid residues, termed N- and C-lobe, each of which contains 14 cysteine residues and forms seven intradomain disulfide bridges. The C-terminal 25-residue stretch of predominantly uncharged and hydrophobic amino acid residues is believed to form the GPI membrane anchor\textsuperscript{125}.

MTf was found to highly accumulate into the mouse brain following intravenous injection\textsuperscript{123, 128}. The studies showed that MTf could cross the brain endothelial cells with a much higher rate than that of transferrin and albumin without affecting the integrity of the BBB. These characteristics of MTf suggest that it could be a good candidate for drug
delivery into brain. It should be noted that these studies were performed with human MTf in mouse brain and bovine brain microvascular endothelial cells, suggesting there is no significant species discrepancy. This is advantageous because it facilitates in vivo study in animal models, and simplifies the translation from animal studies to future human studies.

The underlying mechanism of MTf transcytosis is still unclear, and currently no specific receptor has been identified for MTf. Although MTf shares high extent homology with transferrin, it has been shown that the TfR does not mediate MTf transcytosis\(^{123}\). Instead, a member of LDL receptor family, LDL receptor-related protein (LRP), may play an essential role in this regard \(^{123}\). LRP has also been suggested to mediate the transcytosis of another transferrin family member, lactoferrin, across the BBB\(^{133}\).

**Viral Vectors for Gene Therapy**

A perfect vector system to deliver therapeutic genes should meet several stringent criteria. It should be administrated by a non-invasive route without causing severe damages to the tissue/organ structures of the subject; it should have high specificity and target only desired cells within the target areas; it should express the therapeutic genes at an optimal amount for a defined length of time; it must not cause a severe inflammatory response to compromise the overall health of the subject and the therapeutic effect. In other words, efficiency, specificity, and safety are the three top priorities and golden standards which must be considered for the development of any vector system for clinical
usage. In addition, the vector system per se should be produced by a well established protocol at high efficiency and stability.

Many strategies and vectors are currently being explored to maximize efficiency, specificity, and safety, which include viral vectors, nonviral vectors, and naked DNA. Due to their unique advantages and disadvantages, no single type of vector is suitable for all gene therapy applications at this moment.

Viral vectors, in general, have higher gene transfer efficiency as compared to nonviral vectors due to the viruses’ highly evolved biological machineries to enter host cells and reproduce themselves utilizing the cellular machineries. Regardless of oncolytic viral vectors in cancer gene therapy that are designed to induce cell death of tumor, viral vectors are deprived of some or all of their vital viral coding sequences to minimize vector’s toxicity resulting from viral replication and viral genes’ expression, leaving their cis-acting elements intact for, for example, viral genome packaging into virus capsid. The therapeutic genes, which are of interest, are then cloned into the viral genome in place of the deleted sequences. The deleted genes that are essential and necessary for viral replication and reproduction are compensated by a separate packaging cell line acting as a “helper” in trans to produce complete recombinant viral vectors. For adenoviruses, the produced viral particles are separated and purified from the packaging cells, usually by two cesium chloride density gradients\(^ {134}\). The purified adenoviral vectors are then quantified by plague-forming assay (PFU) as infectious titer and by spectrophotometer as physical viral particle titer\(^ {135}\).

The most abundant viral vectors being used in gene therapy field are adenoviruses, retroviruses (including lentiviruses), poxviruses (including vaccinia viruses), aden-
associated viruses, and herpes simplex viruses. These vectors can be classified into two categories according to whether their viral genomes are permanently integrated into the host chromosomes (retroviruses) or transiently but predominantly persist in cell nucleus (Ad, AAV, and HSV) or cytoplasm (poxviruses). Currently, the integrating viral vector is our first choice when dividing cells are the target. For example, these vectors are used for haematopoietic stem cells (HSCs) in order to treat inborn immune deficiency disorders.\textsuperscript{136} The non-integrating viral vectors are usually utilized to transfect postmitotic, differentiated cells such as hepatocytes or neurons, providing relative short-term transgene expression but minimal risk of oncogenic integration. Among all diseases being treated by gene therapy, curing cancer is still a lofty and insurmountable task. Cancer gene therapy stands for a new paradigm of cancer treatment and accounts for 66\% of gene therapy clinical trials.\textsuperscript{12} Conventional approaches to cancer gene therapy mainly utilize non-replicating viral vectors to deliver therapeutic factors, such as tumor suppressor gene p53 or prodrug activator HSV type 1 (HSV-1) thymidine kinase. In addition, oncolytic viral vectors, which harnesses the cell lytic properties of many viruses under the replication condition, have also been developed. Ad and HSV-1 are being extensively investigated in this regard, and a variety of tumor-selective, replication-competent oncolytic Ads\textsuperscript{138} and HSV-1\textsuperscript{139} have been developed.

Even though viral vectors at present are still the best vehicles for efficient gene delivery to most cell types, their “Achilles heel” greatly limit their clinical utilization. A major problem that gene therapists are facing is the immune responses caused by the virus and the transgene expression. These include 1) the non-specific immunity against pathogens such as cytokine-mediated inflammatory responses and macrophage clearance
effect; 2) specific immune response induced by Ads such as anti-Ad antibodies and cytotoxic T lymphocytes (CTLs); and 3) immune responses against the transgene. For example, following Ad administration, pre-existing immunity against Ads immediately reacts with the viral vectors through neutralizing antibodies to their capsid proteins; pre-existing CTLs can be induced by capsid proteins through MHC-I; infiltrative CTLs can be induced against the gene products of Ads after cells are infected. These immune responses not only hinder the viral vectors to be fully effective, but also compromise the safety of viral vectors as therapeutics to human beings and even cause death if several critical parameters, e.g. dose, are not handled properly. Numerous progress has been made in immune-evasive delivering strategies such as using “gutless” adenoviral vectors that are stripped of all viral genes, which significantly reduces the CTLs and prolongs the expression of carried transgenes.

**Adenovirus**

Among all vector system and delivery strategies used for gene therapy clinical trials, adenoviral vectors are most commonly used tools to date. Ad is a non-enveloped icosahedral particle measuring 70-100nm in diameter, with the protein shell (capsid) encompassing the linear double-stranded genomic DNA in the core. Ad was first isolated from degenerated human adenoids tissues and characterized as a distinct viral agent in 1953, and thus far more than 100 members have been identified in Adenoviridae family. Human adenovirus (Mastadenovirus H) is comprised of 50 serotypes and are classified into 6 subgroups (A-F) based on their unique biological properties. Adenovirus serotype 5 (Ad5) in subgroup C is a very common infectious pathogen to human, which
causes mild cold-like symptoms\textsuperscript{154}. Ad5 is the most frequently used adenoviral vectors and has been widely used, for example, in cancer gene therapy as oncolytic agents, in the treatment of genetic diseases as a gene delivery vector, in vaccine development as an antigen carrier and immunity booster\textsuperscript{141, 155, 156}. Studies of human Ad as a gene delivery vector have focused primarily on the closely related serotype 2 (Ad2) and 5 (Ad5) viruses because of several reasons. First, Ad can infect a variety of cell types at high efficiency, including both dividing and non-dividing cells. Second, efficient and well accepted protocols make virus production and propagation easy in laboratory, and very high titer viral stocks can be obtained by two simple purification steps, which is crucial for \textit{in vivo} studies and clinical trials. Furthermore, the well characterized pathology and an extensive collection of mutants, e.g. replication-incompetent virus, make Ad2 and Ad5 relatively safe agents to fulfill gene delivery. Moreover, remarkable advances have been achieved in Ad vector development with regard to targeting modification, which allows the viral vector to specifically infect certain cell types\textsuperscript{157}, and imaging modification, which allows the viral vector and its associated transgene expression to be dynamically traced and monitored by non-invasive photon-detection technology\textsuperscript{158}, etc. Our studies have focused on Ad5 and its derived mutants, which will be discussed in detail in this dissertation.

Ad5 capsid is composed of more than 7 different protein components, which include three major proteins hexon (II), penton base (III), and fiber (IV) as well as several minor proteins VI, VIII, IX, IIIa as the structural supporters (\textbf{Fig. 1}). The major capsid proteins hexon, penton base and fiber are considered as the 252 capsomeres forming the overall structure of the capsid. Inside the capsid, two terminal proteins (TP) are covalently linked to the double stranded genomic DNA, and the three major core proteins
**Figure 1** A stylized section of Adenovirus type 5 particle based on the current understanding of its protein and DNA components. The major capsid proteins hexon (II), penton base (III), and fiber (IV) form the scaffold of the particle, and minor proteins IIIa, VI, VIII and IX act as cements or stabilizers. The capsid encompasses a double-stranded, linear DNA genome with a length of 35937 bps, which is covalently or noncovalently linked to several core proteins V, VII, X, and TP. The protein IX and the knob domain of fiber are highlighted in red and blue since they are the focuses of this dissertation.
Mu, V, and VII mediate and condense the chromosome conformation through non-covalent interactions with the genome.\textsuperscript{153}

The typical Ad infectious cycle is approximately 20-24 hours long, and can be clearly divided into an early stage and a late stage. The early phase starts from the initial binding of adenovirus on cell surface until the beginning of the replication of the viral DNA, which indicates the start of the late phase. The Ad5 infection begins with the adsorption and entry of the virus into cells. At the very beginning of infection, the fiber-knob protein binds to Ad5’s native cellular receptor, the Coxsackievirus B and Adenovirus Receptor (CAR), whose extracellular portion is composed of two immunoglobulin-like domains providing high affinity binding with the knob domain in the fiber protein.\textsuperscript{153} This step provides enough proximity between the viral particle and cell to allow an arg-gly-asp (RGD) sequence in the penton base protein to interact with integrins on the cell surface, which triggers the internalization event cascade of Ad5 virions. The internalized Ad5 virions in endosomes then undergo endosome escaping, microtubule-mediated cytosol movement, before finally reaching the nuclear pores. During internalization and intracellular trafficking processes, Ad5 particles experience a sequential disassembly process to get rid of its shell proteins and to expose the DNA-core complex.\textsuperscript{153} The DNA-core complex, maybe with a few remaining hexon capsomeres, will be transported into the nucleus compartment for further viral gene transcription and replication.

The Ad5 genome carries five early transcription units, E1A, E1B, E2, E3, and E4, which are located closely to the ends of viral chromosomes, possibly for the purpose of early cis- and trans-activation (\textbf{Fig. 2}). The early transcription of these units serves to
**Figure 2** Transcription of the adenovirus genome. The early transcripts are outlined in green, the late in blue. Arrows indicate the direction of transcription. The gene locations of the VA RNAs are denoted in brown. MLP, Major late promoter. The numbers represent the map units along the adenovirus genome.

provide an optimal environment for viral replication, to antagonize host anti-viral defenses, and to synthesize gene products necessary for viral replication. In particular, E1A encodes two proteins (12S 13S) that activate transcription and induce host cells to enter S phase of the cell cycle by mediating the functions of a variety of cellular factors such as retinoblastoma susceptibility protein (pRB), TATA-binding protein (TBP), E2F, p300, and CBP. E1A proteins also participate in inhibiting the host anti-viral measures of interferon -α –β. E1B encodes two proteins (55kd, 19kd) that block apoptosis through interaction with cellular factors, e.g. through shortening the half life of p53. E1B proteins also contribute to the cell activation along with E1A. E2-encoded proteins (DBP, pTP, and polymerase) directly mediate DNA replication. E3-encoded proteins antagonize host anti-viral responses such as CTLs and apoptosis induced by tumor necrosis factors (TNFs) and Fas ligand. E3-encoded gp19kd also interferes with MHC class I antigen processing. E4-encoded proteins (E4orf 1-6/7) are involved in transcriptional regulation and mRNA transportation, and also mediate DNA replication and cellular apoptosis\textsuperscript{153}.

A variety of redundant mechanisms are discovered in transcription activation and viral functional pathways with different efficiencies within various cell types, which are believed to have evolved to ensure proper infectivity in different cells\textsuperscript{153}.

As the early gene products accumulate, DNA replication activity starts to be observed after 5-8 hours of infection. Two inverted terminal repeats (ITRs) at the chromosome ends, especially the first 18 base pairs, serve as the replication origins, which are recognized by E2-encoded pTP and polymerase to initiate DNA replication. The pTP and polymerase, along with several host cell nuclear factors (NFI, NFII), mediate the subsequent chain elongation reactions\textsuperscript{153}. 
The late stage transcription can be observed after the onset of viral DNA replication. The onset of DNA replication probably changes the conformation of chromatin and permits the transcription factors (Sp1/MAZ) access the major later promoter and activate the late stage transcription by E1A proteins. The late transcription unit, which encodes the majority of structural components and essential elements for the virus maturation, is organized in a single large transcript (about 29kb in length) under the driving of the major late promoter (MLP). The large primary transcript undergoes complicated splicing events during mRNA maturation. The derived mRNAs can be grouped into five different families (L1-L5) based on different poly (A) sites\textsuperscript{153}.

There are two transcription units, IVa2 and minor capsid protein IX, with high transcription activity at the onset of DNA replication and are termed delayed early units. These two delayed early genes play important roles in activating the MLP\textsuperscript{153}.

Along with the accumulation of viral structural proteins in the nucleus, such as hexon and penton capsomeres, assembly of viral progenies begins with the formation of empty capsids, followed by the encapsidation of viral DNA molecules. $10^4$ viral progenies per cell are typically produced with the synthesis of substantial excess of virion protein and DNA. During the late stage, two events facilitate the release and escape of produced viral progenies: disruption of the intermediate filament of cellular skeleton by E1B 19kd and L3 encoded protease, and cell death due to E3 encoded ADP (adenovirus death protein)\textsuperscript{153}. 
Capsid-Modified Adenovirus for Gene Therapy

Wild type adenovirus is far from perfection as a powerful tool in a variety of gene therapy scenarios. For instance, the requirement of access to Ad native receptor CAR for efficient Ad infection greatly limits its gene delivery potential in cells devoid of CAR (Fig. 3). An example is that differentiated airway epithelial cells express CAR and integrin molecules on their basolateral membranes, which make them inaccessible to Ad vector delivered to the apical surface through the airway\textsuperscript{159, 160}. Another example is that human tumor cells frequently express little to none of CAR\textsuperscript{161}, which makes many tumor cells resistant to adenovirus-mediated cancer therapy strategies. To address this issue, strategies are being developed that aim at modifying the adenovirus vector tropism to achieve CAR-independent transduction. Since the knob domain of fiber in Ad capsid mediates the initial binding of Ad with CAR receptor and is the major determinant of Ad tropism, numerous efforts have been focused on the modification this particular protein to achieve Ad tropism alteration (see the review\textsuperscript{47}).

Another challenge to use Ad as the gene therapy vector/tool is that robust immune responses and pre-existing neutralizing antibodies in circulation against adenovirus rapidly clear viral particles and therefore considerably lower the efficiency of Ad vector in terms of gene delivery and expression\textsuperscript{144-146, 162}. Several studies suggest that the neutralizing antibodies against Ad5 appear to be generated principally against the major capsid proteins\textsuperscript{163-165}, particularly against hexon capsomers\textsuperscript{148, 163, 164, 166, 167}. Therefore, many studies sought to overcome the host-anti Ad immune response by modifying major capsid proteins fiber and hexon\textsuperscript{142, 143, 147, 149, 168}. 
Figure 3 The pathway of Ad5 infection. The cell entry of Ad5 starts with the initial high affinity binding of the knob domain of fiber the primary cellular receptor, CAR. The extracellular portion of CAR is comprised of two Ig-like domains providing the high affinity binding. Subsequent internalization of the virus is triggered by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base protein with integrins on the cell surface. After a sequential disassembly of the virion, viral DNA is released and imported into the nucleus.
Moreover, modification of Ad capsid proteins were also explored for the purpose of enhancing the vector’s utility for gene therapy applications. A typical example of this is that: due to the lack of effective and appropriate means to detect and track adenovirus, fluorescence proteins and other imaging modalities were incorporated into the minor capsid proteins IX, which allowed non-invasive detection and tracking of viral localization and replication events\textsuperscript{49-52}. The point in these studies of choosing capsid labeling versus expressing transgenes for functional implementation is that capsid labeling may provide better efficacy in certain circumstance when direct functional display is superior to indirect accumulation of gene product, whose efficacy is limited and affected by environmental factors. In the case of vector imaging, reporter gene expression driven by an exogenous promoter can only provide indirect information of virus localization and does not necessarily coincide with viral replication, and the gene expression level is determined by transcriptional and translational activity of that particular transgene in the target cells. Misreading may also be caused due to non-specific expression in non-target cells. Hence, reporter gene expression cannot be reliably employed to analyze differential biodistribution as a result of specific viral infection. If linked to the Ad capsid, the imaging modality functions directly after viral infection with no need of transcription and translation. This property is especially important for transducing certain cell types where exogenous promoter activity is low.

There are two major strategies to incorporate heterologous peptide ligands on Ad particles: adapter protein-mediated linkage and genetic integration. The adapter-based strategy possesses several advantages. For example, the adapter protein strategy does not engage Ad capsid structural alteration and therefore maintain Ad’s biological advantages
in gene therapy. The adapter protein strategy also grants flexibility of Ad-incorporated heterologous peptides by just modifying the adapter protein. However, the systemic complexity of adapter-based strategy is a major limit of this strategy, e.g. at least two components, vector and peptide ligand, must be produced independently and then conjugated together, which leads to significant variation and might compromise the repeatability and accuracy. Besides, it has been experimentally determined that a very large amount of fusion protein is required for animal studies as well as in clinical settings, and affinity and stability of protein-protein interactions is a major determinant in a successful experimental design. On the other hand, the genetic incorporation strategy builds the vector and functional ligand as a whole, but it is a more difficult and complicated task to fulfill and the delicate structure and biological synthesis pathway of capsid components restricts such genetic incorporation with strict limitations in terms of, for example, size and type\textsuperscript{156, 169}.

**Capsid Protein Fiber: A Potential Locale for Re-targeting Adenovirus**

The splicing events of the major late primary large transcript result in 5 subfamilies of late transcript (L1 - L5). The L5 transcript encodes the polypeptide IV as the structural component of fiber protein. The 3-D structure of fiber protein has been determined by X-ray crystallography\textsuperscript{170} and cryo-EM\textsuperscript{171}. The Ad5 fiber protein is a homotrimer, with its N-terminal domain (40 residues) embedded in the penton base and its C-terminal 180 residues forming a bulb-like domain (i.e. the knob domain) as the major cell binding motif. The knob domain extrudes from each vertex so as to facilitate cell binding events. Between the N terminus and C terminus is a beta-spiral structure
called the shaft domain, which is composed of 22 repeats of 15-residue in β-strand and loop structures. CAR has been determined as the native cellular receptor of Ad5, and has been characterized as a member of the immunoglobulin (Ig) super family. The extracellular domain of CAR is composed of two Ig-like domains (D1 and D2), in which the homodimer D1 is sufficient to provide high affinity binding (Kd = 0.1~20 nM) with fiber knob at the AB loop region of each monomer.

Since the fiber protein mediates the primary and initial cellular binding events and determines the native tropism of Ad5, a number of studies have been performed to modify the fiber protein to alter the native tropism of Ad5 and to re-target Ad5 to other cell surface molecules, e.g. integrins, heparin sulfate proteoglycans (HSPG), CD40, CD46, carcinoembryonic antigen (CEA), human epidermal growth factor receptor type 2 (Her2), etc. Tropism alteration is usually achieved by removing the knob domain to ablate the native tropism, by exchanging the fiber protein with other binding ligands to replace the native tropism, by inserting another binding ligand into the fiber protein or other locale of the viral particle to add a new tropism.

In these studies, two major strategies were adopted to modify fiber proteins. In the first strategy, re-targeting Ad5 vectors is achieved via a bi-specific adapter protein that binds both Ad5 viral particle and the molecules expressed on target cells. The feasibility and efficacy of this strategy has been demonstrated in a number of in vitro and in vivo studies. For example, a bi-specific adaptor protein that is composed of soluble CAR ectodomain (sCAR) and single chain anti-carcinoembryonic antigen (CEA), namely sCAR-MFE, has been shown to re-target Ad5 to CEA-expressing tumors in vivo.
following intra-tumor or intravenous injection\textsuperscript{189}. The result of this study also suggested that the adaptor protein ablated the Ad5 native tropism in liver, most likely by blocking the interaction between fiber and Ad5 native receptor, which is beneficial for targeted gene therapy via systemic delivery route.

The second strategy is to genetically modify the Ad fiber protein through modifying the fiber gene sequence in the Ad genome. Generally, extra targeting ligands are genetically incorporated into the fiber protein to obtain alternative cell entry measures independent of CAR-mediated pathway. Two locales in the fiber knob domain, the HI loop\textsuperscript{169, 186, 201-203} and the C-terminal end\textsuperscript{185, 193}, are found to be feasible for direct insertion of extra cell-binding motifs to achieve CAR-independent gene delivery.

Belousova et al. found that peptides up to 83 amino acid residues can be incorporated into the HI loop with only marginal negative consequences on the key properties of the Ad5 vectors\textsuperscript{169}. However, this study also found that the infectivity and yield of the fiber-modified recombinant virus were negatively correlated with the size of the ligands used for virus modification, indicating a size limit. To overcome this problem, more versatile platforms for the genetic re-targeting strategy have been developed, such as replacing the knob and/or shaft domain of the fiber, have been developed\textsuperscript{181, 183, 184, 204-206}. Relatively large globular protein domains such as TNF-like domain of CD40L (~150 amino acid residues), growth factor (IGF, ~70 aa), a single-chain antibody (~240 aa) or an affibody (~ 60 aa) have been successfully used to genetically modify Ad5 vectors and resulted in selective gene delivery into the cells. Nonetheless only very few types of heterologous ligands can be used in this scenario possibly due to the ligands’ low conformation compatibility with fiber protein during viral particle assembly.
Protein Transduction Domain (PTD) of HIV-1 Tat

Cell penetration peptides (CPPs) represent a group of small cationic peptides (10-30 residues) that can transverse most, if not all, mammalian cell membranes of lipophilic nature. CPPs have highly diversified structures which have very limited sequence similarity (the most common feature of them is the high frequency of highly positive charged residues, such as Arg and Lys), and the mechanisms of their cellular entry are unclear, even though several ones have been proposed, such as endosome- and macropinocytosis-dependent endocytosis. CPPs have been extensively employed to carry biological active compounds (drugs) into cytoplasm and/or nucleus, and the molecular weights of the compounds can be up to several times bigger than those of CPPs themselves. More importantly, a transcellular property was found on certain CPPs that resulted in transcellular delivery of drugs into bystander cells and much higher in vivo efficiency. This property is extremely valuable for delivering therapeutic compounds to surrounding cells near the infusion sites and reaching widespread effect.

The most extensively studied CPP is the protein transduction domain (PTD) of Tat, PTDtat, which is a transcriptional activator protein of HIV-1 and -2 virus. PTDtat usually refers to the 9-11 residues ((YG)RKKRRQRRR) in the middle region that forms a basic domain within Tat, which is involved in nuclear and nucleolar localization as well as RNA binding. Similar to other CPPs, the detailed mechanism of PTDtat cellular entry is unclear. Nonetheless, the consensus is that the Tat protein as well as the PTD-cargo fusion proteins utilize several distinct mechanisms for cellular entry, such as receptor-dependent endocytosis (clathrin- or lipid raft-
associated\textsuperscript{214, 227-229} or receptor-independent pathways (e.g. macropinocytosis\textsuperscript{230, 231}), depending on the overall conformation of the fusion protein, the cell type of target, and other variables of different experimental settings\textsuperscript{214}. For the initial recognition of PTDtat on the cell surface, it is commonly agreed that the ubiquitously distributed heparan sulfate proteoglycans (HSPGs) play a very important or the most important role. HSPGs confer both high affinity and specificity to the cell binding of Tat proteins\textsuperscript{232-235}, which are determined by properties of Tat proteins (charge and structure) and HSPG molecules (size, saccharide composition, and the distribution of sulfation).

Noting the potency of the PTDtat in mediating cellular uptake and transcellular delivery of small and large molecules, a number of studies have been performed to develop novel therapeutic delivery system by fusing the PTDtat into cargo molecules, including proteins, viruses, liposomes and plasmid DNA. The efficacy of this strategy has been demonstrated both \textit{in vitro}, as proof-of-principle studies, and \textit{in vivo}, indicating its strong promise for further translational applications (see the review\textsuperscript{212}). It is noteworthy that intraperitoneally injected h-galactosidase protein fused with PTDtat was found to be translocated into all tissues in mice, including the brain\textsuperscript{236}, suggesting that the blood-brain barrier is permeable to PTDtat. In neurons, the anti-apoptotic Bcl-XL protein plays a critical role in preventing apoptosis during ocular development and pathologic progression\textsuperscript{237-240}. Bcl-XL fused with PTDtat was found to be selectively uptaken by retina ganglion cells, optic nerves and a subset of amacrine cells in the inner nuclear layer after intravitreal infusion\textsuperscript{241, 242}. This is extremely attractive for molecular neuroprotective strategy on RGCs because of their degeneration in glaucoma\textsuperscript{243-245} and retinitis pigmentosa\textsuperscript{246, 247}. These studies strongly suggest that the blood-brain barrier is
permeable to PTDtat, and this system has therapeutic potential for treating ocular diseases.

**Capsid Protein IX: A Potential Locale to Carry Multifunctionalities**

Numerous efforts have been made on Ad modifications in a very broad range of foci, which include incorporation of peptide ligands on a variety of capsid proteins. Tremendous advances have been obtained in last decades with respect to Ad capsid modification\(^{248}\). The large amount of available functional peptide ligands, if chosen and incorporated appropriately, could endow the viral vector with various modalities and enable Ad vectors to act as a multi-modality platform for gene therapy applications.

Genetic incorporation of heterologous polypeptide ligands into adenovirus through the fusion with a structural protein warrants a number of considerations. First, the requirement of surface-exposed configuration confines the heterologous ligands to three major capsid proteins, hexon, penton base and fiber, as well as two minor cement proteins, protein IX and IIIa (Fig. 1). Second, the incorporation efficiency of a structural fusion protein into virions and hence its copy number per virion should be sufficient to deliver an effective dose of the functional ligand. For example, an estimate of 20 copies of fluorophores per virion has been reported for adenoviral particle visualization\(^{249}\). Third, fusion with the candidate protein should minimally perturb its cellular localization as well as its normal function. This requirement would maximize the possibility for incorporation and also preserve the function of the protein for viral infection and replication. In this regard, a common hurdle of placing peptide ligands on three major capsid proteins is that they play a crucial role in capsid assembly and viral stability, and
their delicate structure appeared to be vulnerable to the addition of incorporated peptide ligands, which results in assembly problems and potentially gives rise to less viable Ad virions. Incorporation at these sites apparently has a strict size limitation\textsuperscript{153, 156, 169, 185, 250}. Meanwhile, the minor capsid protein IIIa and IX, termed cement proteins, are thought to stabilize or strengthen the intermolecular interaction of hexons. Dimitriev et al. have demonstrated that both pIIIa and pIX can accommodate C-terminal extensions of heterologous peptides\textsuperscript{251, 252}.

pIX, a 140 amino acids protein (~14.3 kilo dalton), is expressed at delayed early stage and acts as a stabilizing cement via its N-terminal domain interaction\textsuperscript{253-256}. Four trimers of pIX interact with a group of nine hexons (GON) in each facet of the icosahedron\textsuperscript{257}, resulting in 240 copies of the protein per virion\textsuperscript{258, 259}. pIX is considered unnecessary for virus assembly\textsuperscript{260}. However, it is essential for the thermostability of the viral particle and the packaging of full length genomes\textsuperscript{261}. Recent Cryo-EM data\textsuperscript{262}, and structural and functional studies of pIX\textsuperscript{255, 263} suggested a theoretical possibility that the C terminus of pIX, which either binds on the capsid surface or extends outward from the capsid, can tolerate considerable modification and be utilized as an anchor for the addition of heterologous ligands to Ad particles. This thought was further demonstrated by several studies on Ad tropism alteration\textsuperscript{251, 264, 265}, \textit{in vivo} imaging and tracking of physical Ad particles\textsuperscript{49-51} and cancer therapeutics\textsuperscript{52} via genetically incorporating corresponding heterologous polypeptides on pIX C-terminus. In these studies, more importantly, pIX has been shown to tolerate considerably large modification without perturbation of viral viability and capsid stability. For example, a green fluorescent protein (GFP, 238 aa)\textsuperscript{49, 51}, and a HSV thymidine kinase-firefly luciferase fusion protein
have been successfully added to the C terminus of pIX with their native functions maintained. Therefore, pIX would be an attractive candidate as a locale to accommodate heterologous peptide ligands.

In particular, tropism alteration can be achieved via incorporation of targeting ligands on the pIX\textsuperscript{251, 264, 267}. Imaging motifs, such as fluorescent proteins, can also be incorporated on the pIX to provide direct visualization of viral vectors, which is invaluable for \textit{in vivo} tracking\textsuperscript{49-51}. In addition, toxins can be linked to the pIX and delivered to cancer cells for cancer gene therapy\textsuperscript{52, 266}.

Single-chain variable fragment (scFv) is a single peptide fusion of a heavy chain and a light chain of an immunoglobulin variable region that are linked by a flexible peptide. It maintains the antigen specificity of the original immunoglobulin with a greatly reduced molecular weight and is much facile to construct. It has been shown that scFv can be fused with retrovirus envelope protein for targeted infection\textsuperscript{268-272}. Recent evidences have shown that the temporary incorporation of cellular receptor-specific scFv on Ad capsid protein, such as the fiber knob, could redirect Ad vector to a CAR-independent cell entry pathway and achieve cell-specific targeting and gene delivery\textsuperscript{188, 189, 198, 273, 274}.

Nevertheless, scFv, as well as many other polypeptide ligands, is directed to the secretary pathway which is essential for its maturation and native functionality, whereas the Ad capsid proteins are synthesized in the cytoplasm and transported into the nucleus for viral assembly, where the reducing environment prevents formation of scFv disulfide bonds\textsuperscript{275}. The discrepancy in biosynthesis of the two components leads to a possibility that genetically incorporated scFv may not function because of the lack of the incorrect
folding and the lack of post-translational modification. However, some scFvs, which are termed hyper-stable scFv and can be produced in a soluble form in the cytoplasm with their activity retained, have been successfully engineered into fiber and pIX\textsuperscript{183,276}. These studies proved the feasibility that scFv can be genetically incorporated into pIX for targeting purposes.

A scFv specifically recognizing tumor carcinoembryonic antigen (CEA, a relevant tumor marker in ovarian, breast and colon cancer), termed MFE-23, has been shown to be a powerful agent for re-targeting adenovirus to epithelial tumor metastases using an adapter protein\textsuperscript{189}. In addition, a re-engineered derivative of MFE-23, termed hMFE, has been developed with a greatly increased retention time in tumors (~4 days versus 10 min of original) and improved stability\textsuperscript{277}, which greatly enhanced the utility of MFE-23 and expanded its usage into intracellular reducing environment.

Fluorescent proteins on pIX can be utilized to reveal direct localization of Ad vectors. As we mentioned, gene expression in the targeted cells or tissues is a basic requirement for future widespread application of gene therapy. Therefore, a sensitive tool to quantitatively access the gene transfer and expression is required. Until recently, several strategies have been developed for non-invasive imaging of Ad vectors, such as reporter gene expression, which can provide information on the \textit{in vivo} location of gene delivery and magnitude of gene expression. However, as previously mentioned, expression of reporter genes can only provide indirect information of virus localization and replication, which is dependent on transcriptional and translational activity of that particular transgene in the target cells. Misreading may also be caused due to non-specific expression in non-target cells. Hence, reporter gene expression cannot be reliably
employed to analyze differential biodistribution as a result of targeting. If linked on Ad capsid, polypeptides function directly after viral infection with no need for transcription and translation. This property is especially important for transducing certain cell types where promoter activity is low. Researchers have developed several pIX-modified Ad vectors which were genetically labeled with fluorescent proteins, such as GFP and monomeric red fluorescent protein (mRFP1), and exhibited strong potential in viral particle detection both in vitro and in vivo\textsuperscript{49-51}.

The Aequorea jellyfish derived GFP and its blue, cyan, and yellow variants have been widely used as genetically encoded indicators for tracking gene expression and protein localization and as donor acceptor pairs for fluorescence resonance energy transfer (FRET)\textsuperscript{278}. The red fluorescent protein cloned from Discosoma coral (DsRed or drFP583) also holds great promise as a spectrally distinct companion or substitute of GFP. In addition, the tissue penetration of GFP detection is limited to 1-2 mm in experimental animals\textsuperscript{279, 280}, while the red fluorescent proteins with far-red and near infrared spectra would give deeper signal tissue penetration. However, DsRed is an obligate tetramer which usually has a slow and incomplete maturation and hampers its translation from a scientific finding to a generally applicable and robust tool. Its monomeric variant mRFP1 essentially addressed this critical issue and has shown its potential utility in biotechnology and cell biology\textsuperscript{278}.

Herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) and the suicide system based on it have been widely utilized for molecular chemotherapy and cancer gene therapy due to its specific recognition and activation of pro-drugs such as ganciclovir (GCV). GCV is a synthetic analogue of 2’-deoxy-guanosine and is first
phosphorylated to a deoxyguanosine triphosphate (dGTP) analog. This competitively inhibits the incorporation of dGTP into DNA, inhibiting DNA polymerase and inducing chain termination\textsuperscript{281}. The adenoviral vector is one of the most common carriers for HSV-TK expression in suicide gene therapy, and has shown synergistic enhancement in tumor killing due to its oncolytic effect\textsuperscript{282}. 
GENETIC INCORPORATION OF THE PROTEIN TRANSDUCTION DOMAIN OF TAT INTO AD5 FIBER ENHANCES GENE TRANSFER EFFICACY

by

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ABSTRACT

Background: Human adenovirus serotype 5 (Ad5) has been widely explored as gene delivery vector for a variety of diseases. Many target cells, however, express low level of Ad5 native receptor, the Coxsackie-Adenovirus Receptor (CAR), thus are resistant to Ad5 infection. The Protein Transduction Domain of HIV TAT protein, namely PTD_{tat}, has been shown to mediate protein transduction in a wide range of cells. We hypothesize that re-targeting Ad5 vector via PTD_{tat} motif would improve the efficacy of Ad5-mediated gene delivery.

Results: In this study, we genetically incorporated PTD_{tat} motif into the knob domain of Ad5 fiber, and rescued the resultant viral vector, Ad5.PTD_{tat}. Our data showed the modification did not interfere with Ad5 binding to its native receptor CAR, suggesting Ad5 infection via CAR pathway is retained. In addition, we found that Ad5.PTD_{tat} exhibited enhanced gene transfer efficacy in all the cell lines that we have tested so far, which included both low-CAR and high-CAR cells. Competitive inhibition assays suggested the enhanced infectivity of Ad5.PTD_{tat} was mediated by binding of the positively charged PTD_{tat} peptide to the negatively charged epitopes on cell surface. Furthermore, we investigated in vivo gene delivery efficacy of Ad5.PTD_{tat} using subcutaneous tumor models established with glioma U118MG cells, and found that Ad5.PTD_{tat} exhibited enhanced gene transfer efficacy compared to unmodified Ad5 vector as analyzed by non-invasive fluorescence imaging technique.

Conclusion: Genetic incorporation of PTD_{tat} motif into Ad5 fiber allowed Ad5 vectors to infect cells via the alternative PTD_{tat} targeting motif while retaining the native CAR-mediated infection. The enhanced infectivity was demonstrated in both cultured cells and
tumor models. Taken together, our study identified a novel tropism expanded Ad5 vector that may be useful for gene therapy applications.

**BACKGROUND**

Human adenovirus serotype 5 (Ad5) has been widely explored as gene delivery vector, largely owing to its superior gene delivery efficacy, minor pathological effect on humans, and easy manipulation *in vitro*. Several problems, however, have been identified in the course of development and application of Ad5-based gene therapy protocols, one of which is the inefficient gene delivery into target cells\(^1\text{--}^3\). It is known that infection of Ad5 is initiated by attachment of its capsid fiber protein to the cell surface coxsackie virus adenovirus receptor (CAR), which is followed by interaction of penton base with \(\alpha_v\) integrins that triggers the internalization of the viruses\(^4\text{--}^7\). Many target cells, such as malignant tumor cells, are found to express very low level of CAR, thus are resistant to Ad5 infection. Therefore, strategies to redirect Ad5 infection via alternative receptors would be useful for gene therapy applications.

Since fiber, the capsid protein extruding from Ad virion surface, is an essential mediator of Ad5 infection, fiber modification has been explored as a means to re-direct Ad5 tropism. Ad5 fiber is composed of an N-terminal tail that is attached to penton base on the virion surface, a shaft domain consisting of 22 repeats of 15-amino acid residue motif, and a C-terminal globular domain, namely knob, which functions as a receptor binding domain. Because of the essential role of the fiber knob domain in mediating Ad5 infection, knob modification could be one of the most effective ways to re-direct Ad5 tropism. Indeed, both genetic and non-genetic strategies have been shown to successfully
retarget Ad5 vectors. For example, bi-specific adapter proteins that bind both the knob domain and the alternative receptors expressed on the surface of the target cells have been employed to re-direct Ad5 infection\textsuperscript{8-11}. In addition, genetic incorporation of RGD peptide and/or polylsine epitope into the knob domain allowed Ad5 to infect cells through alternative receptors (cell surface integrins for RGD and negatively charged epitopes such as heparan sulfate proteoglycans for polylsine), thus greatly improved the gene delivery efficacy Ad5 vectors in many target cells\textsuperscript{12-15}.

Protein transduction domains (PTD) or Cell Penetrating Peptides (CPP) are a class of small peptides that can traverse the plasma membrane of many, if not all, mammalian cells\textsuperscript{16-20}. Among these peptides, the PTD of the Tat protein, namely PTD\textsubscript{tat} hereafter, of human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2) has been one of the most widely studied PTDs. PTD\textsubscript{tat} consists of 11 highly basic amino acid residues, \textit{YGRKKRRQRRR}\textsuperscript{21, 22}. The mechanism of how PTD\textsubscript{tat} crosses the cell membrane has been of intensive study, but controversies remain\textsuperscript{23-26}. Nonetheless, it is commonly agreed that the interaction between the positive charge of the PTD domain and the negative epitopes, in particular, the heparan sulfate proteoglycans expressed on cell membrane plays an essential role in the internalization of PTD\textsubscript{tat} fusion proteins\textsuperscript{17, 20, 27}. Further studies suggest that interaction between PTD\textsubscript{tat} and heparin/heparan sulfate is specified by both charge and structure of the peptide and the proteoglycans\textsuperscript{17, 27-30}.

Given the potential importance of the PTDs in drug delivery, much interest have been attracted in exploiting this system as a tool to deliver therapeutic molecules or particles into mammalian cells. PTDs have been widely used in the field of protein therapy, in which PTDs are fused to the protein of interest, and used to deliver the heterologous
protein into cultured cells\textsuperscript{17, 20, 31}. More interestingly, it has been demonstrated in several mouse studies that PTD\textsubscript{tat} fusion proteins can be delivered into different tissues \textit{in vivo} following systemic administration, and therapeutic benefits have been observed\textsuperscript{32-35}. In addition, PTDs have been used to deliver other large molecules or particles such as plasmids, liposomes, nanoparticles, phages and viruses, with variable efficiency\textsuperscript{36-41}. In these applications, PTDs were conjugated to the particles by incubation with the particles in coupling solutions. In other words, the coating of the particles is not based on genetic modification, but on ionic or other interactions between the peptides and the particles. Because of the potency of PTD\textsubscript{tat} in mediating cellular uptake of small and large molecules, in this study, we attempted to re-direct Ad5 infection via PTD\textsubscript{tat} pathway. Previous studies have demonstrated pre-treatment of Ad particles with chemically synthesized PTDs or bi-specific adaptor proteins composed of the extracellular domain of CAR and PTDs improved Ad infection\textsuperscript{37, 42}. Nonetheless, intrinsic to these non-genetic modification strategies, the efficiency of retargeting depends on the affinity and stability of protein-protein interactions, thus may be highly variable in different systems. In addition, a large amount of peptides or adaptor proteins will be required for \textit{in vivo} investigations. Our study was designed to retarget Ad5 vectors to PTD\textsubscript{tat} pathway using genetic capsid modification strategy. We genetically incorporated the sequences encoding PTD\textsubscript{tat} peptide into the 3’ end Ad5 fiber gene, rescued the modified viruses, and characterized the viruses in detail. Our data demonstrated that genetic modification of Ad5 fiber with PTD\textsubscript{tat} motif greatly improved the efficacy of gene delivery in both cultured cells and tumor models. Our study thus identified a novel tropism expanded Ad5
vector that may be useful for gene therapy applications, especially for applications involving gene delivery into low-CAR expressing cells.

**RESULTS**

**Development of PTD\textsubscript{tat}-modified Ad5 vector—Ad5.PTD\textsubscript{tat}**

As the receptor binding domain, knob of Ad5 fiber has been shown to be an effective locale for incorporating foreign targeting motifs\textsuperscript{12-15}. In this study, we genetically incorporated PTD\textsubscript{tat} epitope into the C-terminal end of the fiber knob domain (Fig. 1). Ad5 genome contains about 36 kilobases (Kb) and is too large for direct modification using conventional cloning techniques. To achieve our goal, we established a bacteria-based homologous recombination system for Ad5 fiber modification\textsuperscript{15}. Using this system, the sequences encoding PTD\textsubscript{tat} were incorporated into the 3’ end of fiber gene, immediately before the stop code. The modified Ad5 genome (namely Ad5.PTD\textsubscript{tat}) and the unmodified control (Ad5) were both replication deficient because their E1 region, which is essential for Ad5 replication, was replaced with CMV promoter-driven green fluorescence protein (GFP) as reporter gene. The viruses were rescued in 293 cells stably expressing Ad-E1 genes, and purified with CsCl gradient ultracentrifugation. The yield of Ad5.PTD\textsubscript{tat} viruses is in the same range as unmodified Ad5 viruses, suggesting the modification did not interfere with virus formation (data not shown). The modification was confirmed by both polymerase chain reaction (PCR) and sequence analysis of the modified region of the viral genome using viral DNA from purified Ad5 and Ad5.PTD\textsubscript{tat} viruses (data not shown).
**Figure 1** Diagram of PTDtat modified Ad5 vector. (A) PTDtat peptide incorporated into the fiber knob domain. (B) Structural diagram of Ad5 and Ad5.PTDtat vector. The PTDtat motif was incorporated at the C-terminal end of the fiber.
CAR-binding activity of Ad5.PTD_{tat} 

Unmodified Ad5 viruses interact with their native receptor CAR via the fiber knob domain. We thus examined whether incorporation of PTD_{tat} into the knob domain interfered with the Ad5-CAR interaction. An enzyme-linked immunosorbent assay (ELISA) was employed in this regard. In the assay, Ad5.PTD_{tat} or Ad5 viral particles were immobilized in the wells of 96-well maxi-sorp plate, and incubated with various amounts of recombinant extracellular domain of CAR (sCAR) protein. After extensive washing, binding of sCAR to the viruses were assessed by anti-CAR antibody and corresponding secondary antibody conjugated to alkaline phosphatase (AP). The OD405 readings resulted from color reaction with AP substrate represent the binding activity of sCAR to the viruses. As shown in Fig. 2, binding of sCAR to Ad5.PTD_{tat} is similar to that of unmodified Ad5, suggesting the genetically modified vector Ad5.PTD_{tat} maintained its ability to interact with Ad5 native receptor CAR.

Cell-binding activities of Ad5.PTD_{tat} 

The fiber knob domain is responsible for Ad5 binding to the target cells, which is the initial step of viral infection. Ad5.PTD_{tat} was designed to re-direct Ad5 infection. We thus examined whether PTD_{tat} modification had any effect on Ad5 binding to cells. It is known that Ad5 particles maintain their ability to bind to cell surfaces at 4°C while their internalization is completely inhibited. We therefore incubated Ad5.PTD_{tat} or Ad5 with cells expressing different levels of CAR at 4°C for 1 hour, then collected the cells and examined the bound viral particles by quantitative PCR assays that assessing the viral genome copies. We found that Ad5.PTD_{tat} exhibited higher cell-binding activities in
Figure 2 Ad5.PTDtat showed similar CAR-binding activity to unmodified Ad5 vector in an ELISA-based binding assay. In the experiment, 109 VPs of each viral vector were immobilized in the wells of a 96-well ELISA plate, and incubated with increasing concentrations of recombinant sCAR (extracellular domain of CAR, that is, soluble CAR). The binding activity was detected by AP activity conjugated on the detection antibodies.
almost all of the cells we examined, including both high-CAR and low-CAR cells. Shown in Fig. 3 are results obtained in the two representative cell lines: high-CAR expressing HeLa cells, and low-CAR expressing U118MG cells\textsuperscript{43,44}.

**Enhanced gene transfer efficacy of Ad5.PTD\textsubscript{tat}**

We further investigated the gene transfer efficacy of Ad5.PTD\textsubscript{tat} in a variety of cultured cells using the reporter GFP protein. Ad5.PTD\textsubscript{tat} vector or unmodified Ad5 was used to infect cells at different multiplicities of infection (MOIs). Two days after infection, we evaluated the transgene expression using a fluorescent microscope and a fluorescent plate reader. We found that Ad5.PTD\textsubscript{tat} showed more efficient gene delivery than unmodified Ad5 in all of the cells we tested (Fig. 4). In particular, Ad5.PTD\textsubscript{tat} exhibited significant higher gene transfer efficacy than unmodified Ad5 in the cells expressing low or medium level of CAR such as RD cells, U118MG cells, or D65MG cells\textsuperscript{43,44}. In high-CAR cells that are readily accessible to unmodified Ad5 vector, Ad5.PTD\textsubscript{tat} also showed enhanced infectivity, presumably because Ad5.PTD\textsubscript{tat} maintained the CAR-mediated infection pathway while gaining extra targeting activity through PTD\textsubscript{tat} pathway.

**Identification of pathways mediating Ad5.PTD\textsubscript{tat} infection**

Ad5.PTD\textsubscript{tat} showed enhanced gene delivery efficacy compared to unmodified Ad5 vectors. To confirm this expanded tropism was mediated by the genetically incorporated targeting motif PTD\textsubscript{tat}, we performed gene transfer assay in the presence of competitive inhibitors. It has been shown that interaction between the positively charged PTD\textsubscript{tat} and the negatively charged cell surface epitopes such as heparan sulfate proteoglycans is
Figure 3 PTDr modification promoted Ad5 binding to cell surfaces. Binding of Ad5 and Ad5.PTDtat were examined in both high-CAR HeLa cells (A) and low-CAR U118MG cells (B) at 4°C. The viruses associated with the cells were quantified by quantitative PCR after DNA isolation from the cell lysate, and the viral copy numbers were normalized with actin DNA in the samples. The * indicates $p<0.05$ and ** indicates $p<0.01$ as analyzed by Student’s t-test.
Figure 4 Ad5.PTDtat exhibited enhanced gene transfer efficacy in a variety of tumor cells. The gene transfer efficacy was evaluated with the GFP reporter that was carried in the E1 region of each vector. In the assay, tumor cells expressing various levels of CAR were infected with either Ad5 or Ad5.PTDtat at MOI 100 or 500 VPs/cell, and GFP expression were examined with a fluorescence microscope and a fluorescence plate reader. (A) Representative fluorescence images of low-CAR (RD) cells, medium-CAR (D65MG) cells and high-CAR (HeLa) cells that were infected with Ad5 or Ad5.PTDtat at MOI of 500 VPs/cell. (B) GFP expression in a variety of cells infected with either Ad5 or Ad5.PTDtat quantified in a fluorescence plate reader (company).
essential for $\text{PTD}_{\text{tat}}$ mediated protein transduction. Heparin, the structural analogue of heparan sulfate, is thus expected to inhibit $\text{PTD}_{\text{tat}}$ mediated infection. In addition, recombinant knob protein was used to block the native CAR-mediated Ad5 infection because it compete with Ad5 vectors for cell surface CAR. In low-CAR U118MG cells, due to the paucity of CAR, unmodified Ad5 showed poor gene transfer efficacy, and neither knob nor heparin had any effect on Ad5-mediated transgene expression (Fig. 5A). In contrast, Ad5.$\text{PTD}_{\text{tat}}$ exhibited efficient gene delivery into U118MG cells, which was completely inhibited by heparin, but not by the recombinant knob protein (Fig. 5A). These data demonstrated Ad5.$\text{PTD}_{\text{tat}}$ infected low-CAR cells mainly through the incorporated $\text{PTD}_{\text{tat}}$ motif. In high-CAR A549 cells, infection of unmodified Ad5 was completely blocked by recombinant knob protein while heparin had little effect, confirming that unmodified Ad5 mainly infected cells through CAR pathway (Fig. 5B). On the other hand, Ad5.$\text{PTD}_{\text{tat}}$-mediated gene transfer was partially blocked by either knob or heparin, but completely blocked in the presence of both knob and heparin, suggesting Ad5.$\text{PTD}_{\text{tat}}$ could infect cells via both CAR and $\text{PTD}_{\text{tat}}$ motif (Fig. 5B).

**In vivo gene transfer efficacy of Ad5.$\text{PTD}_{\text{tat}}$**

We next examined whether the infectivity-enhanced vector Ad5.$\text{PTD}_{\text{tat}}$ could result in enhanced gene transfer efficacy *in vivo*. Since Ad5.$\text{PTD}_{\text{tat}}$ showed more profound infectivity enhancement for low-CAR tumor cells *in vitro*, we assessed the *in vivo* gene delivery efficacy of the Ad5 vectors using tumor models established with low-CAR U118MG cells. After the tumors were established subcutaneously in athymic nude mice, PBS, unmodified Ad5, or Ad5.$\text{PTD}_{\text{tat}}$ vectors were injected into the tumors. The gene
Figure 5 Competitive inhibition assay showing the enhanced gene transfer efficacy of Ad5.PTDtat was mediated by the PTDtat motif. In the assay, recombinant knob protein (50 ug/ml) was used to block CAR-mediated viral infection, and heparin (100 ug/ml) was used to block PTDtat mediated infection. Infections were performed at MOI of 100 VPs/cell. (A) In low-CAR U118MG cells that are resistant to unmodified Ad5 vector, Ad5.PTDtat mediated efficient gene delivery and the efficacy was completely inhibited by heparin, while recombinant knob had little effect, suggesting the enhanced infectivity of Ad5.PTDtat in low-CAR cells resulted from the PTDtat motif. (B) In high-CAR A549 cells, Ad5.PTDtat mediated gene delivery was partially inhibited by either knob or heparin, but completely inhibited in the presence of both inhibitors, suggesting Ad5.PTDtat infected high-CAR cells via both CAR and PTDtat pathways.
delivery efficacy of each vector was analyzed by non-invasive fluorescence imaging that detects GFP expression in live mice. As shown in Fig. 6A, Ad5.PTD\textsubscript{tat}-infected tumors showed more intensive green fluorescent signals than Ad5-infected tumors, while no signal was detected in PBS-injected tumors. Quantitative analysis of the green fluorescence signals revealed that Ad5.PTD\textsubscript{tat}-mediated GFP expression was significantly higher than that of unmodified Ad5 vector in the tumors ($p<0.01$) (Fig. 6B). These data suggest the infectivity-enhanced Ad5.PTD\textsubscript{tat} vector could be a useful vector for \textit{in vivo} gene delivery into tumors, which is essential for cancer gene therapy.

**DISCUSSION**

In this study, we sought to improve the gene transfer efficacy of Ad 5 vectors by genetic modification of the fiber knob domain with PTD\textsubscript{tat} motif. Our data demonstrated the success of this strategy. The fiber modified Ad5 vector, Ad5.PTD\textsubscript{tat}, not only exhibited enhanced gene delivery efficiency of Ad5 vectors in low-CAR cells that are resistant to unmodified Ad5 infection, but also in high-CAR cells that are permissive to Ad5 infection. The enhanced infectivity of Ad5.PTD\textsubscript{tat} was found to be mediated by targeting of PTD\textsubscript{tat} to the negatively charged epitopes on cell surface such as heparan sulfate containing proteoglycans. In addition, we found PTD\textsubscript{tat} mediated Ad5.PTD\textsubscript{tat} infection is additive to native CAR-mediated infection as assessed by competitive inhibition assays, which is not unexpected since Ad5.PTD\textsubscript{tat} maintained full CAR-binding activity. More significantly, the enhanced gene delivery efficacy of Ad5.PTD\textsubscript{tat} was demonstrated \textit{in vivo} using low-CAR U118MG tumor models, and employment of a recently developed
Figure 6 PTDtat modification of Ad5 fiber enhanced in vivo gene delivery efficacy of the vector. In vivo gene delivery of Ad5.PTDtat was examined using non-invasive fluorescence imaging technique in low-CAR tumor models. 1010 VPs of Ad5 or Ad5.PTDtat were injected into the subcutaneous U118MG tumors, and in vivo green fluorescence images were acquired at different days post viral injection. (A) Representative in vivo images from PBS, Ad5, or Ad5.PTDtat injected mouse tumor models at day 7 after vector administration. The colors representing different intensity of signals are shown on the color bar. Ad5.PTDtat infection resulted in more intensive GFP signals than unmodified Ad5 vectors. (B) Quantitative analysis of the GFP intensity in the tumor models of each group. The * marks significant differences (p<0.01) as analyzed by Student’s t-test.
non-invasive optical imaging system allowed us to visually detect the enhanced gene delivery in vivo.

As a cell penetrating peptide, PTD\textsubscript{tat} is capable of traversing the plasma membrane of mammalian cells. Since the initial description that PTD\textsubscript{tat} is responsible for the ability of HIV Tat protein to enter mammalian cells, PTD\textsubscript{tat} has attracted tremendous interest as a drug delivery vehicle\textsuperscript{16-20}. Further interest has been stimulated by the observations that PTDs can facilitate systemic delivery of biologically active recombinant proteins in vivo\textsuperscript{32-35, 37}. Since inefficient gene delivery into target cells has been one of the major limitations in Ad5-mediated gene therapy, in this study, we attempted to employ PTD\textsubscript{tat} peptide to facilitate Ad5 mediated gene delivery. Employment of PTDs to facilitate virus infection has been investigated previously, but only using non-genetic methods\textsuperscript{37, 42}. In particular, chemically synthesized PTDs (PTD\textsubscript{tat} and PTD\textsubscript{antp}) or bi-specific adaptor proteins consisting PTDs and the extracellular domain of CAR have been used to coat Ad vectors and resulted in enhanced gene delivery\textsuperscript{37, 42}. Compared to the non-genetic methods, our genetically PTD\textsubscript{tat} modified vector is advantageous in two major means: 1) genetic modification allows stable interaction between Ad5 and the PTD\textsubscript{tat} targeting epitope, thus reducing the volatility associated with the affinity and stability of protein-protein interactions in the presence of different environmental factors, especially for in vivo applications; and 2) genetic modification does not require production of peptides or fusion proteins other than the viral vector, while large amount and high quality of protein/peptide production is required for non-genetic strategies (in addition to high quality production of the viral vectors), which is especially important for in vivo studies.
One issue associated with PTD<sub>tat</sub>-mediated protein delivery is the inefficient release of PTD<sub>tat</sub> fusion proteins from the endosomal compartments<sup>24, 45-48</sup>. It has been demonstrated that a large portion of PTD<sub>tat</sub> fusion proteins remain trapped in non-cytosolic compartments even though they are efficiently taken up by the cells, which apparently would compromise the therapeutic effect of the fusion protein. In our study, we examined the trafficking of the internalized Ad5.PTD<sub>tat</sub> particles by immunofluorescent staining of the particles, and found Ad5.PTD<sub>tat</sub> were transported inside the cells in a similar way to unmodified Ad5 vectors (data not shown), suggesting endosomal trapping is not significant, if any, for the infection of Ad5.PTD<sub>tat</sub> viruses. In addition, the enhanced gene delivery mediated by Ad5.PTD<sub>tat</sub> confirmed that the virions efficiently escaped the endosomal compartments.

The potential utility of the infectivity-enhanced Ad5.PTD<sub>tat</sub> vector in cancer gene therapy was initially investigated in this study using low-CAR expressing tumor models. Indeed, many tumor cells have been shown to express very low levels of CAR, which is partially responsible for the low efficacy of Ad5 mediated cancer gene therapy in in vivo studies, especially in clinical trials<sup>1-3</sup>. The ability of Ad5.PTD<sub>tat</sub> to improve the gene delivery efficacy is attributable to the PTD<sub>tat</sub> motif, which binds to the negatively charged motifs expressed on cell surface, in particular, heparan sulfate containing proteoglycans that are widely expressed in a variety of cells including tumor cells. In addition to cancer gene therapy, Ad5.PTD<sub>tat</sub> may also be applied in other gene therapy applications where infectivity-enhancement is beneficial. Infectivity-enhanced vectors will not only allow efficient gene delivery into low-CAR target cells, but also allow use of less viral vectors, thus reducing vector-associated toxicity.
Previous studies have developed several other infectivity-enhanced vectors, which include Ad5 modified with RGD, polylysine, or knobs from other serotypes of adenoviruses\textsuperscript{13-15, 49}. Since each of the modified vectors uses a unique extra targeting motif, the enhanced gene delivery efficacy in a specific cell type depends on the expression of individual receptors on the cell surface. Similar to PTD_{tat}, the polylysine epitope, which is composed of a stretch of lysine residues, is highly basic, and can utilize the heparan sulfate as receptor. Nonetheless, interaction between PTD_{tat} and heparan sulfate is not only based on ionic interactions, but also on the specific structures of the peptide and the proteoglycans\textsuperscript{27-29}. Therefore, choice of an infectivity-enhanced vector needs to be determined for a specific application involving gene delivery enhancement.

CONCLUSIONS

Our data show that genetic modification of Ad5 fiber knob domain with PTD_{tat} did not interfere with binding of fiber to its native receptor CAR. The modified vector, Ad5.PTD_{tat}, delivered transgenes into both high-CAR and low-CAR cells more efficiently than the unmodified Ad5 vector. Our data further show Ad5.PTD_{tat} infected cells via both CAR and PTD_{tat} pathways. More significantly, Ad5.PTD_{tat} exhibited enhanced gene delivery \textit{in vivo} in tumor models, thus may be useful for gene therapy applications involving low gene delivery efficacy.

METHODS

Cell culture
The human embryonic kidney 293 cells stably transformed with Ad-E1 DNA, human lung carcinoma A549 cells, human cervix adenocarcinoma HeLa cells, human embryonic rhabdomyosarcoma RD cells, and human glioma D65MG and U118MG cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The 293 cells, A549 cells and U118MG cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (DMEM/F12) containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine. HeLa cells were cultured in minimum essential Eagle medium (MEM) containing 10% FBS and 2 mM L-glutamine. Both RD and D65MG cells were cultured in DMEM containing 10% FBS and 2 mM L-glutamine. All of the cells were maintained at 37°C in a 5% CO₂ humidified incubator.

**Generation of Ad5.PTDₜₜ vector**

Genetic modification of Ad5 vector with PTDₜₜ was achieved using our previously established fiber modification system\(^{15}\). In brief, the fiber shuttle vector containing a unique SnaB I restriction site immediately in front of the stop code of fiber gene, namely pNEB.PK.SnaBI, was used to generate PTDₜₜ modification. The sense and antisense oligonucleotides encoding PTDₜₜ motif, 5’-phos-ACT TTT TCA TAC ATT GCG CAA GAA GGC GGT GGA GGG TAT GGC AGG AAG AAG CGG AGA CAG  CGA CGA AGA TAA TAA A-3’ (sense) and  5’-phos-TTT ATT ATC T TTA GCT GTC GCT GTC TCC GCT TCT TCC TGC CAT ACC CTC CAC CGC CTT GGT CAA TGT ATG AAA AAG T -3’ (antisense), were annealed and cloned into the fiber shuttle vector pNEB.PK.SnaBI, and resulted in a fiber modified shuttle vector pNEB.PK.PTDₜₜ. In order to incorporate the modified fiber into Ad5 genome, pNEB.PK.PTDₜₜ was linearized.
and recombined in *Escherichia coli* (E. coli) BJ5183 with linearized Ad5 backbone plasmid pVK50 that contained CMV promoter driven GFP reporter gene in its E1 region. After the positive recombinant plasmid, designated pAd5.PTD_{tat}, was identified, stable and high quality of the plasmid was obtained from E. coli DH5α after re-transformation of the construct. The modification was confirmed by sequencing analysis.

The modified virus Ad5.PTD_{tat} was rescued and purified as previously described. In brief, the pAd5.PTD_{tat} plasmid was digested with Pac I (to release the viral genome), purified, and transfected into 293 cells stably expressing the complementary E1 genes. After the virus plaques formed, they were amplified in 293 cells, and purified with a standard CsCl gradient protocol. The viral particle (VP) titer was determined using a conversion factor of $1.1 \times 10^{12}$ VPs per absorbance unit at 260 nm.

**ELISA**

The ELISA binding assay was performed essentially as described. In brief, $10^9$ VPs of either Ad5 or Ad5.PTD_{tat} in 100 ul of 100 mM carbonate buffer (pH 9.5) was immobilized in each well of a 96-well maxisorp plate (Nunc, Roskilde, Denmark) by overnight incubation at 4°C. Following extensive washes with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-Tween), and blocking with 2% bovine serum albumin (BSA) in TBS-Tween, the viruses were incubated with various amounts of purified recombinant sCAR. The binding of sCAR to the viruses was detected by incubation with anti-CAR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), followed by alkaline phosphatase (AP)-conjugated secondary antibody incubation. AP activity reflecting the bound amount of sCAR was determined using color reaction with p-
nitrophenyl phosphate (Sigma, St. Louis, MO) as recommended by the manufacturer. The absorbance at 405 nm (OD405) was obtained with PowerWaveHT 340 microplate reader (BioTek Instruments Inc., Winooski, VT).

**Cell binding assay**

Cells were cultured in 6-well plates until they were confluent. The plate was then cooled down on ice, and incubated with Ad5 or Ad5.PTD_{tat} at MOI=5000 for one hour at 4°C. After washing cells twice with cold phosphate buffered saline (PBS) on ice, the cells were collected by incubation with Versene (0.53 mM EDTA). After two more washes with PBS, the cells were lysed and processed to DNA isolation (Qiagen Inc., Valencia, CA). The viral copy number in the DNA samples were obtained by quantitative PCR using primers designed for the E4 region of adenoviral genome. The data were normalized with actin DNA in each sample.

**Gene transfer assay**

Gene transfer efficacy of the viral vectors was assessed with the GFP reporter. In the assay, cells were plated in 24-well plates with a density of 10^5 cells per well the day before infection. Then the cells were infected with Ad5 or Ad5.PTD_{tat} at MOIs of 100 or 500 as described previously^{50}. Two days later, GFP expression was examined by fluorescence microscopy and quantified with Synergy HT fluorescence plate reader (BioTek Instruments Inc., Winooski, VT).

**Competitive inhibition assays**
Low-CAR U118MG cells or high-CAR A549 cells were plated in 24-well plates at a density of $10^5$ cells per well the day before infection. Viruses equivalent to MOI of 100 were used for each infection. To block cell surface CAR, recombinant knob protein was pre-incubated with cells at final concentration of 50 ug/ml prior to viral infection\textsuperscript{51}, and to block the PTD\textsubscript{tat} epitope, the viruses were pre-incubated with 100 ug/ml of heparin\textsuperscript{15,51}. Two hours after infection, the cells were washed with PBS, and refreshed with complete media containing 10% FBS. The cells were cultured for two days in the humidified 37°C, and 5% CO\textsubscript{2} incubator, and GFP microscopy was performed to examine the transgene expression.

\textit{In vivo gene delivery}

The subcutaneous tumors were established in athymic nude mice using $1\times10^7$ U118MG cells per tumor per mouse. After the tumors developed to ~0.5 cm in diameter, PBS or $10^{10}$ VPs of Ad5 or Ad.PTD\textsubscript{tat} were injected into each tumor (n=6). GFP expression was analyzed at 3, 7, and 10 days post infection using a custom-built non-invasive optical imaging system described previously\textsuperscript{52}. The mice were placed in the imaging chamber under anesthesia with 3% isoflurane. Green fluorescence images were acquired at f/8 with 20-second exposure using a combination of excitation filter HQ487/15× and emission filter D535/30m (Chroma Technology, Rockingham, VT). with WinView32 software (Roper Scientific Inc., Trenton, NJ). All of the procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and performed according to their guidelines.
COMPETING INTERESTS

The authors declare that they have no competing interests in relation to this manuscript.

AUTHORS’ CONTRIBUTIONS

TH participated in the generation and in vitro characterization of the adenoviral vectors. YT carried out in vitro and in vivo gene transfer assays. HU performed immunohistochemistry studies. LEP participated in cell culture and tumor model establishment. GPS helped in immunohistochemical studies and in the preparation of the manuscript. JLC assisted in the design of the study and manuscript preparation. HW conceived of the study, participated in its design and coordination, and drafted the manuscript. All authors read and approved the final manuscript.

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REFERENCES


DIRECTING ADENO VIRUS ACROSS THE BLOOD-BRAIN BARRIER VIA MELANOTRANSFERRIN (P97) TRANSCYTOSIS PATHWAY IN AN IN VITRO MODEL

by

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SUMMARY

Adenovirus serotype 5 (Ad5) is widely used in the development of gene therapy protocols. However, current gene therapy strategies involving brain are mostly based on intra-cranial injection. A major obstacle for systemically administered vectors to infect brain tissue is the blood-brain barrier (BBB). One strategy to cross the BBB is transcytosis, a transcellular transport process that shuttles a molecule from one side of the cell to the other side. Recently, melanotransferrin (MTf)/P97 was found to be able to cross the BBB and accumulate in brain. We thus hypothesize that re-directing Ad5 vectors to the MTf transcytosis pathway may facilitate Ad5 vectors to cross the BBB. To test this hypothesis, we constructed a bi-specific adaptor protein containing the extracellular domain of the coxsackie-adenovirus receptor (CAR) and the full-length melanotransferrin (sCAR-MTf), and investigated its ability to re-direct Ad5 vectors to the MTf transcytosis pathway. We found this adaptor protein could re-direct Ad5 to the MTf transcytosis pathway in an in vitro BBB model, and the transcytosed Ad5 viral particles retained their native infectivity. The sCAR-MTf mediated Ad5 transcytosis was temperature- and dose-dependent. In addition, we examined the directionality of sCAR-MTf mediated Ad5 transcytosis, and found the efficiency of apical-to-basal transcytosis was much higher than that of basal-to-apical direction, supporting a role of this strategy in transporting Ad5 vectors towards the brain. Taken together, our study demonstrated that re-directing Ad5 to the MTf transcytosis pathway could facilitate gene delivery across the BBB in vitro.

Key words: adenovirus, melanotransferrin, transcytosis, blood-brain barrier, gene therapy.
INTRODUCTION

Human adenovirus serotype 5 (Ad5) is widely explored as a gene delivery vector for a variety of tissues/organs including brain, largely owing to its high gene delivery efficiency, easy manipulation, high titer production in vitro, well-characterized biology, and minor pathological effect in humans. However, for brain-related gene therapy strategies, such as brain cancer gene therapy, and gene therapy for genetic diseases involving central nervous system pathology, current protocols under development are mainly based on intra-cranial injections.\textsuperscript{1-3} This is not only clinically difficult because it requires high risk surgical procedures, but also inefficient in gene delivery since localized injections do not allow Ad5 vectors to access all diseased cells. Therefore, it is essential to develop a strategy that allows systemically administered Ad5 vectors to infect the brain cells.

A major obstacle for intravascular-administered Ad5 vectors to enter the brain cells is the blood-brain barrier (BBB).\textsuperscript{2-3} The BBB is formed by brain capillary endothelial cells, which are closely sealed by tight junctions and contain few fenestrae and few endocytic vesicles. The BBB restricts the transport of most small polar molecules and macromolecules from the cerebrovascular circulation into the brain tissue, including therapeutic agents including adenoviral vectors.

One strategy to cross the endothelial barrier is transcytosis, a transcellular transport process that shuttles a molecule from one side of the cell to the other side. It involves endocytosis, intracellular transport, and exocytosis of the molecule. Typical examples of this pathway include the transport of albumin and lipids, hormones, and peptides that bind avidly to albumin,\textsuperscript{4-5} and transferrin receptor (TfR) mediated
transcytosis.\textsuperscript{6-7} Transcytosis pathways are not only employed by blood components, but also adopted by viruses such as human immunodeficient viruses, adeno-associated virus and poliovirus.\textsuperscript{8-10}

Recent studies discovered that human melanotransferrin (MTf, also named P97), a transferrin homolog originally identified in human melanoma, was highly accumulated into the mouse brain following intravenous injection.\textsuperscript{11-12} These studies demonstrated that MTf could cross the brain endothelial cells without affecting the integrity of the BBB and with a much higher rate than what is seen with transferrin and albumin.\textsuperscript{11-12} These studies suggest that MTf could be a good candidate for drug delivery to brain. It should be noted that these studies were performed with human MTf in mouse brain, and in \textit{in vitro} BBB models established with bovine brain microvascular endothelial cells (BBMVEC), suggesting MTf functions without significant species discrepancy.

MTf is a glycosylated protein, and exists in two forms including a cell membrane bound form and a soluble, secreted form.\textsuperscript{13} The exact function of both forms remains largely unknown. The membrane bound MTf is composed of 719 amino acid residues, which comprises two homologous extracellular domains of 342 and 352 amino acid residues respectively, and a C-terminal 25-residue stretch of predominantly uncharged and hydrophobic amino acid residues which is believed to form a glycosylphosphatidylinositol (GPI) membrane anchor.\textsuperscript{13} It has been demonstrated that transendothelial transport of MTf occurs via receptor-mediated endocytosis, and low-density lipoprotein (LDL) receptor-related protein (LRP) appears to be involved in MTf transendothelial transport.\textsuperscript{11}
In this study, we attempted to re-direct Ad5 vectors to the MTf transcytosis pathway so that they can deliver transgenes across the BBB in an *in vitro* model system. Infection of Ad5 is initiated by attachment of its capsid protein fiber to the cell surface coxsackie-adenovirus receptor (CAR), followed by interaction of another capsid protein, penton base, with cell surface $\alpha_v$ integrins that triggers the internalization of the viruses.\textsuperscript{14-16} Based on this information, investigators have utilized bi-specific adaptor proteins that bind to both Ad5 vectors and alternative receptors expressed on the surface of the target cells to re-direct Ad5 tropism.\textsuperscript{17-19} In our study, we attempted to re-direct Ad5 across the BBB using the bi-specific adaptor protein strategy. We constructed a bi-specific adaptor protein containing the extracellular domain of CAR (sCAR) and the full-length MTf, namely sCAR-MTf, and demonstrated its ability to re-direct Ad5 vectors across the BBB using an *in vitro* BBB model system. This work represents the first study that employs transcytosis pathway to re-direct Ad5 vectors across the BBB.

RESULTS

**Generation of bi-specific adaptor protein sCAR-MTf.** Previous studies have shown that bi-specific adaptor proteins that bind both Ad5 vector and alternative receptors can be used to re-direct Ad5 vector entering cells through the alternative receptors.\textsuperscript{17-19} In order to re-direct Ad5 vector to MTf transcytosis pathway, we constructed a bi-specific adaptor protein containing the extracellular domain of CAR and the GPI anchor-deleted full-length MTf (termed sCAR-MTf) (Fig. 1A). In the adaptor protein, sCAR that binds to Ad5 fiber is located at the N-terminal end, while MTf that targets to MTf transcytosis pathway is located at the C-terminal part. The GPI-deleted
MTf was employed because deletion of the GPI anchor has been shown to allow production of the soluble form of MTf. In addition, a bacteriophage T4 fibritin trimerization domain was used to connect the two binding domains since fiber exists as trimer in Ad5 virions and trimerized sCAR is expected to bind fiber better than sCAR monomer. Two His\textsubscript{6} epitopes (in the middle and at the C-terminal end) were included to ensure protein purification. Similarly, a control protein sCAR that contains the sCAR, His\textsubscript{6}, and fibritin trimerization domains, but not MTf domain was constructed. The expression of the adaptor proteins, which were driven by CMV promoter, was accomplished using Ad5 as gene delivery vector in mammalian HeLa cells as described in the methods and materials. Proteins were purified from the media since the adaptor proteins were designed as secreted proteins, and confirmed by western blotting analysis using both anti-CAR and anti-MTf antibodies (Fig. 1B).

Next, we examined whether the adaptor proteins retained their ability to interact with Ad5 vectors. In vitro ELISA binding assay was performed in this regard. In the assay, unmodified Ad5 viral particles were immobilized to each well of a 96-well ELISA plate, and incubated with different amount of fusion protein sCAR-MTf or control sCAR. After extensive wash, the binding activity was detected with anti-His\textsubscript{6} antibody, followed by incubation with alkaline phosphatase (AP)-conjugated secondary antibody. The binding activity was assessed based on the AP activity. The results indicated that sCAR-MTf was capable of binding to Ad5 vectors, and the maximal binding could reach ~80% of that between sCAR and Ad5 vectors (Fig. 1C).

**Ad5 transcytosis mediated by sCAR-MTf.** To test whether the bi-specific sCAR-MTf adaptor protein was able to re-direct Ad5 to MTf transcytosis pathway, we
Figure 1 Generation of bi-specific adaptor protein sCAR-MTf. (A) Diagram of the bi-specific adaptor protein sCAR-MTf and control sCAR. H6: His6 epitope; Fbr: T4 Fribritin trimerization domain. (B) Western blotting assay showing the adaptor proteins were expressed and purified. In the assay, about 500 ng of each purified protein were used for SDS-PAGE and subsequent western blotting assay with anti-CAR and/or anti-MTf antibody. Note: the upper fainter band in sCAR lane appears to be the trimeric form of sCAR. We often observed this when a large amount of protein was loaded. (C) ELISA binding assay suggesting sCAR-MTf can efficiently bind to Ad5 vectors. In the assay, Ad5 vectors were immobilized on the wells of a 96-well ELISA plate, and then incubated with different amount of sCAR or sCAR-MTf. The binding activity was analyzed by anti-His6 antibody.
employed an in vitro BBB model system that has been widely used. The BBB model was established with BBMVEC cells in a transwell system, in which the BBMVEC cells were grown on the transwell inserts (polyester, 3.0 um pore size, 12mm diameter) (Fig. 2A). To establish a well-sealed endothelial barrier, the cells were cultured for 5-7 days following confluency, with media refreshed every other day. In the meantime, the transendothelial electrical resistance (TEER) was monitored. TEER reflects impedance of the passage of small ions through the physiological barrier, and has been widely used to measure BBB integrity. Transcytosis assays were performed when the TEER of each BBB model reached the maximal value, which mostly were in the range of 100-150 Ω·cm².

Since previous studies have shown that MTf prefers apical-to-basal transcellular transport, in the transcytosis assay, we pre-mixed Ad5 viral particles with sCAR-MTf or control sCAR, and added the complexes to the upper chamber (apical side of the barrier). The cells were then incubated for 6 hours in a 37°C, 5% CO₂-containing humidified incubator to allow transcytosis to occur. At the end of the experiments, we collected the samples from both the apical and basal chambers, and analyzed the presence of Ad5 virions and adaptor proteins. In addition, we also collected the BBMVEC cells by trypsin treatment to analyze the presence of Ad5 viral particles that were endocytosed and remained inside the cells. Presence of the Ad5 particles was examined with quantitative real-time PCR that measures the Ad5 E4 copy number. Western blotting assay was performed to detect the presence of the adaptor proteins after concentrating the samples using protein concentrator. As shown in Figure 2, we found that in the presence of sCAR-MTf, over 50 times more Ad5 particles, which accounted for about 5% of total
Figure 2 sCAR-MTf mediated Ad5 transcytosis in the in vitro BBB model. (A) Diagram of the in vitro BBB model. BBMVEC cells were cultured on the transwell inserts. (B) Ad5 vectors were re-directed to transendothelial transport pathway in the presence of sCAR-MTf. Following transcytosis assay, samples were collected from both the apical and basal chambers. The BBMVEC cells were also collected by trypsin treatment. DNA isolation was performed for each sample and processed for quantitative PCR to detect Ad5 copy numbers using primers in the E4 region. Apical-to-basal transendothelial transport of Ad5 viral particles were significantly increased in the presence of sCAR-MTf, suggesting Ad5 vectors were re-directed to transcytosis pathway by sCAR-MTf. The * indicates $p<0.01$, and # indicates $p>0.05$, as analyzed by Student’s t test. (C) Western blotting assay showing sCAR-MTf, but not sCAR, was transported to the basal chamber together with the Ad5 vectors, which further confirmed the transendothelial transport of the Ad5 vectors was mediated by sCAR-MTf.
Ad5 particles, were transported to the basal chambers compared to that in the presence of sCAR (p<0.01) (Fig. 2B). Consistent with this, we detected sCAR-MTf protein in the basal chamber samples, but not sCAR, although similar amounts of adaptor proteins were detected in the upper chambers, which indicated that similar amounts of the proteins were used in the assay (Fig. 2C). Furthermore, our results showed that the amount of Ad5 particles that were endocytosed and remained inside the cells in the presence of sCAR-MTf was similar to that of in the presence of sCAR (p=0.42), and was significantly less than what was transcytosed to the basal chamber in the presence of sCAR-MTf (p<0.01) (Fig. 2B). This suggests most Ad5 vectors were re-directed by sCAR-MTf to transcytosis pathway, not endocytosis pathway.

It should be noted that we have repeated the experiments many times, and often there were 40-50 times more transcytosed Ad5 particles in the presence of sCAR-MTf than that of sCAR, but the range could be as low as 10 times higher or as high as 100 times higher. The variation was probably due to variable cell culture/differentiation conditions and different batches of purified proteins. In addition, following the transcytosis assay, the TEER of each BBB model was checked and no significant difference from prior to the assay was found (Table 1), suggesting the BBB integrity was maintained during the transcytosis process. Together our data suggested the bi-specific adaptor protein sCAR-MTf was capable of re-directing Ad5 vectors to the transcytosis pathway.

**Functionality of the transcytosed Ad5 viral particles.** We further investigated whether the transcytosed Ad5 viral particles maintained their functional integrity during the transcytosis process. We evaluated the functionality of these Ad5 particles
Table 1 A representative set of TEER data before and after transcytosis assay. Three wells were used in the transcytosis assay for each group. Student’s t test was used to determine the p value of the TEER before and after transcytosis. P<0.05 was considered statistically significant.

Abbreviations: sCAR-MTf, soluble coxsackie-adenovirus receptor and the full-length melanotransferrin; TEER, transendothelial electrical resistance.

<table>
<thead>
<tr>
<th>Group</th>
<th>TEER (Ω.cm²) Before transcytosis</th>
<th>TEER (Ω.cm²) After transcytosis</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>Ad5+sCAR</td>
<td>124 ± 8.5</td>
<td>113 ± 12.2</td>
<td>0.27</td>
</tr>
<tr>
<td>Ad5+sCAR-MTf</td>
<td>130 ± 15.0</td>
<td>112 ± 4.7</td>
<td>0.13</td>
</tr>
</tbody>
</table>
Figure 3 Infectivity of the transcytosed Ad5 viral particles. Fifty microliters of each sample obtained from the basal chambers in the transcytosis assay were used to infect A549 cells. The actual MOIs of the infections as shown were calculated based on the corresponding quantitative PCR results. Freshly thawed Ad5 vectors that did not go through transcytosis assay were used to infect cells at MOI of 1, 10, and 100 as positive control, whereas uninfected cells (MOI=0) as negative control. The relative light units (RLU) per mg protein indicating the activity of the luciferase reporter that was incorporated in the Ad5 E1 region suggest the transcytosed Ad5 viral particles maintained their infectivity/gene transfer efficacy.
by examining their gene transfer efficacy. In the experiments, 50 ul of each sample collected from the basal chambers after transcytosis assay were used to infect a high-CAR cell line A549 cells, and their gene transfer efficacy was evaluated using the luciferase reporter gene that was incorporated in the E1 region of the Ad5 vectors. The actual MOIs were calculated based on the Ad5 copy numbers in the transcytosed samples which were obtained by quantitative real-time PCR. Ad5 vectors that did not undergo transcytosis assay were used at various MOIs as control. Our results showed that the transcytosed Ad5 viral particles retained their gene transfer efficacy (Fig. 3), suggesting the transcytosis process did not harm the integrity of the Ad5 viruses.

**Temperature-dependence of sCAR-MTf mediated Ad5 transcytosis.** Transcytosis is an active transcellular transport process. Temperature-dependence is thus one characteristic of transcytosis. This temperature-dependence was demonstrated for recombinant MTf transcytosis. To confirm Ad5 vectors were indeed transported across the endothelial barrier via transcytosis pathway, we examined temperature-dependence of this process by performing the transcytosis assay at either 4°C or 37°C. As shown in Figure 4A, sCAR-MTf mediated transcytosis was completely inhibited at 4°C (p<0.01), while in the control experiment with sCAR, there was no significant difference between 4°C and 37°C (p>0.05).

**Dose-dependence of sCAR-MTf-mediated Ad5 transcytosis.** MTf transcytosis is a receptor-mediated process, sCAR-MTf-mediated Ad5 transcytosis is thus expected to be dose-dependent, and may be saturated. To test this and to determine the optimal dose for the transcytosis assay, we performed transcytosis assays using different amount of sCAR-MTf protein (Fig. 4B). Fixed amount of Ad5 viruses (10^8 VPs) were used in each
Figure 4 Characterization of the temperature- and dose-dependence of sCAR-MTf mediated Ad5 transcytosis. (A) The transcytosis assay was performed at either 4°C or 37°C as described above. sCAR-MTf mediated Ad5 transcytosis was completely inhibited at 4°C. The * indicates p<0.01, and # indicates p>0.05, as assessed by Student’s t test. (B) The transcytosis assay was performed with fixed amount of Ad5 (10^8 VPs) and various amount of sCAR-MTf protein at 37°C for 6 hours as described above. Transendothelial transport of Ad5 was saturated when more than 1.2 ug of the adaptor protein were used.
Figure 5 Directionality of sCAR-MTf mediated Ad5 transcytosis. Ad5 (10^8 VPs) and sCAR-MTf protein (1.0 ug) were pre-mixed and added to either the apical or the basal chamber of each transwell containing the BBB model. Presence of Ad5 viral particles in the opposite chamber was examined by quantitative PCR. The * indicates p<0.01, and # indicates p>0.05, as assessed by Student’s t test.
assay. After the transcytosis assay, the samples from the lower chambers were collected, viral DNA was isolated and subsequently used for quantitative PCR analysis. Our results suggested that efficacy of sCAR-MTf-mediated Ad5 transcellular transport was dependent on the dose of the adaptor protein, and the process was saturated in the presence of 1.2 ug sCAR-MTf adaptor protein when $10^8$ Ad5 VPs were used (Fig. 4B).

**Directionality of sCAR-MTf mediated Ad5 transcytosis.** Recombinant MTf has been shown to prefer apical-to-basal transcytosis, although basal-to-apical could also occur.\(^{11}\) To examine whether the sCAR-MTf adaptor protein maintained the apical-to-basal preference, which is essential for our purpose of directing Ad5 vectors into the brain, we performed transcytosis assay in both directions using the same amount of Ad5 vectors and the adaptor proteins. We did not detect significant basal-to-apical transcytosis of Ad5 vectors in the presence of sCAR-MTf since the amount of transcytosed viral particles was not significantly different from that of control experiment using sCAR, although sCAR-MTf mediated Ad5 transcytosis occurred efficiently in apical-to-basal direction (Fig. 5). These results suggest the adaptor protein has strong apical-to-basal preference in directing Ad5 vectors across the endothelial barrier, supporting its potential role of transporting Ad5 vectors towards the brain.

**DISCUSSION**

In this study, we attempted to re-target Ad5 vectors to MTf transcytosis pathway so that Ad5 vectors can traverse the BBB. We designed and constructed a bi-specific adaptor protein sCAR-MTf, and examined its ability to re-direct Ad5 across the BBB using an *in vitro* BBB model system established with BBMVEC cells. The adaptor
protein was able to re-direct Ad5 vectors to traverse the BBB, often with an efficiency of 40-50 times higher than that of control. The Ad5 viral particles undergone transcellular transport maintained their functionality/infectivity, as assessed by their gene transfer efficacy. In addition, the adaptor protein mediated Ad5 transcytosis was temperature- and dose-dependent, which are the characteristics of receptor-mediated transcytosis, in accordance with previous studies.\textsuperscript{11-12} Importantly, sCAR-MTf mediated Ad5 transcytosis showed strong apical-to-basal preference, arguing for its potential utility in transporting Ad5 vectors into brain tissue.

Employment of an \textit{in vitro} model is essential to study whether the bi-specific adaptor protein sCAR-MTf was capable of re-directing Ad5 vectors traverse the BBB. In this work, the BBB model was established with BBMVEC cells that were obtained from Cell Applications, Inc, at second passage (P2), and used at P≤6. The maximal TEER of each BBB model was often in the range of 100-150 $\Omega\cdot\text{cm}^2$, which was reached around 5-7 days following confluency. There was no significant change in TEER when we cultured the BBB model up to 10 days after the cells became confluent. The TEER values of the BBB model in our study are comparable to other studies using bovine brain microvascular endothelial cells (BMVEC),\textsuperscript{22, 26} similar to rat BMVEC cells,\textsuperscript{24} slightly lower than human BMVEC,\textsuperscript{21} but higher than feline BMVEC.\textsuperscript{23} Clearly, the TEER is different for different cell types. In addition, it has been shown that TEER can be modulated by many factors such as cytokines, cAMP, cGMP, nitric oxide, heat, and co-culturing with other cells.\textsuperscript{21-22, 24-26}

Currently, no specific receptor has been identified for MTf. Although human MTf shares 39% homology with human serum transferrin,\textsuperscript{13} TfR is found not to be responsible...
for MTf transcytosis. Instead, a member of LDL receptor family, LRP, may play an essential role in this regard. In fact, LRP may be a common mediator for its binding partners to traverse the BBB. In addition to MTf, LDL, lactoferrin and LDL receptor-associated protein (RAP) have been found to cross the BBB with high efficiency and the receptor involved in their transcellular transport appears to be LRP. In vivo transport across the BBB of these proteins may also help explain the observations that MTf, lactoferrin, and LRP accumulate in the brain of patients with neurological diseases such as Alzheimer’s disease. Interestingly, LRP-mediated transcytosis may only be a feature of endothelial cells, since in other cell types or organs, majority of these proteins, once internalized, are found to be degraded or recycled. Differentiation stage of endothelial cells also appears to play a role in determining what pathway the protein uptake is taken. For example, in growing brain capillary endothelial cells, LDL is classically internalized by the clathrin pathway, and directed to lysosomes for degradation. However, when the cells are fully differentiated, even though the classic degradation pathway (via lysosomes) is functional, LDL is mostly directed to nondegradation transcytosis pathway. In accordance, our data showed that in the presence of sCAR-MTf, the majority of internalized Ad5 viral particles were directed to the transcytosis pathway in the BBB model that is formed by differentiated cells, and very little remained inside the cells (Fig. 2B). Apparently the transcytosis pathway adopts a different trafficking mechanism from the classical endocytosis pathway since it can bypass the lysosomal degradation. In this regard, caveolae, a type of vesicles that contain enriched caveolin and are non-clathrin coated, has been implicated in LRP-mediated LDL
transcytosis through the brain microvascular endothelial cells.\textsuperscript{27} The precise mechanism for transcytosis of the proteins across the BBB, however, remains to be investigated.

Adenovirus retargeting has been widely explored in terms of specific and effective gene delivery into certain target cells. It has been demonstrated that genetic incorporation of alternative targeting motifs into Ad5 fiber improves gene transfer efficacy and specificity.\textsuperscript{38-41} However, genetic incorporation has a size limit. Epitopes less than 100 amino acid residues may be incorporated into fiber knob domain without affecting viral assembly.\textsuperscript{42} Alternatively, bi-specific adaptor proteins composed of sCAR and the alternate targeting motif such as EGF, hTf and a single-chain antibody against carcinoembryonic antigen have been successfully employed to retarget Ad5 vectors.\textsuperscript{17-19} In vivo stability of this strategy has also been demonstrated.\textsuperscript{43-44} Our study adopted this adaptor strategy since melanotransferrin is a large protein, and the domain responsible for its transcytosis is not defined thus far.

In our study, about 5\% of Ad5 particles could be transcytosed across the BBB in the presence of sCAR-MTf. The percentage, however, may vary from 3\% to 10\% depending on cell condition and the batches of proteins. In addition, we observed about 0.1\% Ad5 transendothelial transport in all of the negative control transcytosis assays, which include experiments with sCAR (Fig. 2, 4A and 5) or Ad5 alone (Fig. 4B). These apparently represented non-specific Ad5 crossing the BBB, presumably due to leakiness through the paracellular space or occasional escape of endosomes and exocytosis to the other side of the barrier.

Systemic gene delivery into brain is one of the most challenging problems faced by gene therapy investigators. The highly defensive BBB turns out to be the major hurdle.
Engineering gene delivery vector so that it can traverse the BBB is thus a rational and attractive direction. In this study, we took advantage of the high efficiency of MTf transcytosis, and applied it in combination with Ad5 vector re-targeting strategy. Another potential advantage using sCAR-MTf for gene delivery into brain is that the major components of the adaptor protein, the extracellular domain of CAR and MTf, are endogenously expressed in human, therefore, the host immune response against the adaptor protein sCAR-MTf is expected to be minimal for \textit{in vivo} applications. Although the data presented here were obtained \textit{in vitro}, the proof-of-principle study appears to be very promising. \textit{In vivo} utility of sCAR adaptor protein mediated Ad5 retargeting strategy has been directly explored and demonstrated in an earlier study, in which an adaptor protein composed of sCAR and anti-carcinoembryonic antigen single chain antibody, sCAR-MFE, has been shown to re-direct Ad5 to the lungs of a transient transgenic mouse model over-expressing carcinoembryonic antigen in the pulmonary vasculature.\cite{19} Our further efforts will be focused on investigation of the \textit{in vivo} utility of sCAR-MTf mediated Ad5 transcytosis, and the means of improving the efficiency of this strategy.

**MATERIALS AND METHODS**

**Antibodies.** The rabbit polyclonal antibody against CAR was purchased from Santa Cruz Biotechnology, Inc. The mouse anti-His-Tag monoclonal antibody (Penta.His) was purchased from Qiagen. To generate mouse monoclonal antibody against MTf, M-19 hybridoma cells that were raised against P97 antigen (that is, MTf) were purchased from American Type Culture Collection (ATCC). Antibody generation and purification were accomplished in the hybridoma core facility at University of Alabama at Birmingham.
Secondary antibodies including Alkaline Phosphatase (AP)-conjugated donkey anti-mouse and Horse Radish Peroxidase (HRP)-conjugated donkey anti-rabbit antibody were purchased from Jackson ImmunoResearch Laboratories, Inc.

**Cells and Cell culture.** The human embryonic kidney cells transformed with Ad5-E1 DNA (293), the human lung carcinoma cell line A549, and the human melanoma cell line MeWo cells were purchased from American Type Culture Collection (ATCC). All of these cells were cultured in Dulbecco’s modified Eagle medium-Ham’s F12 medium (DMEM/F12) that contains 10% fetal calf serum (FCS) and 2 mM L-Glutamine, and grown in a 37°C, 5% CO2 humidified incubator. The BBMVEC cells were purchased from Cell Applications, Inc, and cultured in the complete BBMVEC growth media (also from Cell Applications, Inc). The cell culture flasks or transwell inserts were coated with attachment factor solution (Cell Applications, Inc) prior to cell seeding, as described in the manufacturer’s protocol. The cells were maintained in a 37°C, 5% CO2 humidified incubator, and the culture media were refreshed every other day until experimental execution.

**Generation of Ad5 vector that expresses sCAR-MTf fusion protein.** In order to express sCAR-MTf fusion protein using Ad5 vector, we first generated a shuttle vector containing the expression cassette, which is composed of (in order) CMV promoter, sCAR ectodomain that consists of its own leader sequence (amino acid residues 1-236), a 5-amino acid residue peptide link (GGPGS), a His6 epitope in the middle, a bacteriophage T4 fibritin trimerization domain, full-length soluble MTf, and a C-terminal His6 epitope. Construction of this cassette was done sequentially as described below. First, an extra His6 epitope was cloned into the parent vector pcDNA3sCAR6hfibritin
containing sCAR ectodomain, the peptide link (GGPGS), a His$_6$ epitope, and T4 fibrin trimerization domain,$^{45}$ to generate pcDNA3sCAR6hf6h, in which the extra His$_6$ epitope was introduced into the C-terminal end of the expression cassette. The expression cassette then contained all components designed except the MTf fragment. This cassette including the CMV promoter and SV40pA was then amplified by PCR, and subcloned into pShuttle (Stratagene) vector, resulting in pShuttle.sCAR6hf6h.

Next, we obtained human MTf cDNA from human melanoma cell line MeWo cells. This was accomplished by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Briefly, the total RNA from MeWo cells was extracted using Qiagen RNeasy mini kit according to the manufacturer’s manual. The RT reaction was then performed with universal primer oligo dT, and about 1 ug of total RNA was used as template. The full-length soluble form of MTf lacking the C-terminal GPI anchor (27 amino acid residues) was subsequently amplified using the RT reaction as template. This MTf full-length fragment was then inserted into pShuttle.sCAR6hf6h in-frame at the position of between the fibrin trimerization domain and the C-terminal His$_6$ epitope. The resultant plasmid was named pShuttle.sCAR-MTf.

To incorporate the expression cassette of sCAR-MTf into the Ad5 vector, homologous recombination was performed between pShuttle.sCAR-MTf and Adeasy vector (Stratagene). This was accomplished by co-transformation of the linearized shuttle vector pShuttle.sCAR-MTf and backbone pAdeasy into *Escherichia coli* BJ5183. The recombinants were initially screened by DNA isolation and restriction digestions. The plasmid DNA of positive candidates were then transformed into bacteria DH10B, and
more DNA was isolated and screened. The final positive recombinants were confirmed by sequencing analysis, and the resultant Ad5 vector was named pAdeasy.sCAR-MTf.

To rescue the viruses encoding sCAR-MTf, pAdeasy.sCAR-MTf was digested with Pac I, and transfected into the Ad-E1 expressing 293 cells with Superfect (Qiagen). After plaques were formed, they were collected and processed for large-scale amplification in 293 cells. The viruses (named Ad5.sCAR-MTf) were then purified by standard CsCl gradient centrifugation.\textsuperscript{46}

**Protein expression and purification.** We employed HeLa cells to express and purify the adaptor protein. Twenty flasks (185-cm\textsuperscript{2}) of HeLa cells that were grown to 80% confluency were infected with the Ad5 viruses encoding the adaptor protein at multiplicity of infection (MOI) of 500. The infected cells were then cultured for 48 hours in a 37°C, 5% CO\textsubscript{2} humidified incubator prior to protein purification. Since the adaptor protein was designed as secreted protein, we collected the culture media for protein purification. Purification of the protein was performed based on the His\textsubscript{6} epitopes that were incorporated in the adaptor protein, and the Qiaexpressionist\textsuperscript{TM} system (Qiagen) was used to purify the His\textsubscript{6}-tagged protein. For purification, we first concentrated the media to ~ 50 ml using protein concentrator columns (10-KDa or 50-KDa MWCO, Millipore) according to the manufacturer’s manual. Then, 1/10 volume of 10x supplemental buffer (500 mM NaH2PO4, pH 8.0, 1.5 M NaCl, 100 mM imidazole) was added to the media to adjust salt and pH condition of the sample. Next, 1 ml of Ni-NTA agarose (Qiagen) that was pre-washed with phosphate-buffered saline (PBS) was added into the media and the sample was incubated at 4°C for 2 hours on an end-over-end shaker. The Ni-NTA agarose beads were then collected by centrifuging the sample at
1000 rpm for 5 minutes. After washing the beads twice with washing buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, 0.05% Tween-20, pH 8.0), the adaptor protein was eluted with elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole, 0.05% Tween-20, pH 8.0). The protein concentration was determined using Bio-Rad DC protein assay kit.

**Enzyme-Linked Immunoabsorbent Assay (ELISA).** *In vitro* ELISA binding assay was performed similarly as described previously.\(^{39}\) In brief, $10^9$ Viral Particles of Ad5 was immobilized on wells of a 96-well ELISA plate (Maxisorp; Nunc, Roskilde, Denmark) by overnight incubation at 4°C. The wells were then washed four times with Tris-buffered saline (TBS) containing 0.05% Tween 20, blocked with blocking solution (2% bovine serum albumin (BSA) + 0.05% Tween 20 in TBS) for 1 hour, and incubated with different concentrations of purified adaptor proteins in binding buffer (0.5% BSA + 0.05% Tween 20 in TBS) overnight at 4°C. The binding of adaptor protein to Ad5 viruses was detected by incubating with anti-His tag antibody and alkaline phosphatase (AP)-conjugated corresponding secondary antibody, followed by color reaction that detects AP activity. The color reaction was performed using $p$-nitrophenyl phosphate (sigma) as recommended by the manufacturer, and absorbance at 405 nm (OD$_{405}$) was obtained using a microplate reader (Molecular Devices, Menlo Park, CA).

**Western blotting assay.** In the assay, protein samples were boiled in Laemmli sample buffer, separated on 4-15% polyacrylamide gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was then incubated with blocking solution containing 5% skim milk and 0.05% Tween 20 in TBS for 1 hour at
room temperature and processed for incubation with primary antibody against CAR or MTf. After washing with TBS and re-blocking, the membrane was incubated with horse-radish peroxidase (HRP)-conjugated secondary antibody for 2 hours at room temperature. After extensive washing, the immunoreactive bands were detected by ECL plus western blotting detection system as recommended by the manufacturer (Amersham Biosciences).

**In vitro BBB model and transcytosis assay.** The *in vitro* BBB model was established by culturing BBMVEC cells in a transwell system, in which the BBMVEC cells were grown on the transwell inserts (polyester, 3.0 um pore size and 12 mm diameter, Costar). To establish a well-sealed endothelial barrier, the cells were continued culturing for 5-7 days after confluency, with media refreshed every other day. In the meantime, the transendothelial electrical resistance (TEER) was monitored using millicell-ERS apparatus (Millipore). Since the TEER is inversely proportional to the area of the tissue, the standard practice is to report TEER as the product of the resistance (Ω) and the growth area (cm²). In this study, the TEER of each BBB was obtained by subtracting the background resistance (inserts without cells) from the measured barrier resistance, then multiplying by the growth area of 12-mm inserts (1.13 cm²). Transcytosis assay was performed when TEER reached 100-150 Ω·cm², the maximal TEER the BBB models could reach in our study.

The transcytosis assay was performed as following, which was modified from previous studies. In the assay, the BBMVEC cells grown in the transwell system were pre-incubated in Ringer-HEPES solution for two hours at 37°C, 5% CO2. The virus-adaptor protein complex was formed by mixing 10⁸ viral particles (VPs) of Ad5 with 1 ug of adaptor proteins in 250 ul of Ringer-HEPES solution and incubating at 37°C
for 15 minutes. The virus-protein complex was then added to the apical chamber of each well containing the BBB model, in which the basal chamber contains 700 ul of Ringer-HEPES solution. The cells were incubated again in a 37°C, 5% CO$_2$ humidified incubator for 6 hours. At the end of the experiments, the samples from basal chambers were collected to analyze transcytosed viral particles and proteins. Samples from the apical chambers and cell layers were also collected and analyzed.

To analyze the presence of viral particles, 200 ul of the samples were used to extract DNA, and processed for real-time quantitative PCR that assessed the Ad5 E4 copy number. For western blotting assay, the samples were concentrated to about 100 ul with protein concentrator (10-KDa MWCO, Vivascience), then processed for western blotting assay as described above.

**Gene transfer assay.** Gene transfer efficacy of the transcytosed Ad5 vectors was assessed in A549 cells by measuring luciferase activity, essentially as described previously.$^{46}$ In brief, 50 ul of each sample from the basal chambers after transcytosis assay were diluted in 100 ul culture media containing 2% FCS, and used to infect A549 cells plated in a 48-well cell culture plate. Two hours later, 150 ul of complete culture media containing 10% FCS were added into each well, and the cells were continued in culture for 24 hours in a 37°C, 5% CO$_2$ humidified incubator. To measure the luciferase activity, the cells were washed with PBS, and lysed by one freeze-thaw cycle in 100 ul of reporter lysis buffer (Promega). Ten microliters of each sample were used to measure the luciferase activity using a luciferase assay kit (Promega) and a luminometer (Berthold, Gaithersburg, MD). The total amount of protein in each sample was determined by Bro-Rad DC protein Assay kit (Bio-Rad).
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DERIVATION OF A TRIPLE MOSAIC ADENOVIRUS BASED ON MODIFICATION OF THE MINOR CAPSID PROTEIN IX

by

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ABSTRACT

Adenoviral capsid protein IX (pIX) has been shown to be a potential locale to insert targeting, imaging-related and therapeutic modalities by genetic modification. Recent evidences suggested that capsid protein mosaicism could be a promising strategy for improving the utility of Ad vector. In this study, we explored a method to genetically generate triple pIX mosaic Ad serotype 5 (Ad5) displaying three types of pIX on a single virion. pIXs were modified at their carboxy termini with a Flag sequence, a hexahistidine sequence (His$_6$) or a monomeric red fluorescent protein (mRFP1), respectively. Western blotting analysis and fluorescence microscopy of the purified recombinant viruses indicated that all three modified pIXs were incorporated into the viral particles. Immuno-gold electron microscopy (EM) further confirmed that three types of pIX indeed co-existed on an individual virion. These results firstly validated a triple mosaic capsid configuration on pIX, and demonstrated the possibility of further radical design.

KEYWORDS

Adenovirus; triple mosaic Ad; pIX modification

INTRODUCTION

Adenovirus (Ad) is about 70-100 nm in size, non-enveloped icosahedral virus with a capsid of 252 capsomers: 240 hexons forming the faces and 12 pentons at the vertices, each of which bears a slender fiber. Ad genome is linear, non-segmented double stranded DNA with a length of 30-36kb. Ad vectors have been utilized as a basic tool for gene delivery in a variety of studies, and account for one fourth total number of
gene therapy clinical trials worldwide thus far (http://www.wiley.com/legacy/wiley-chi/genmed/clinical/). The broad usage of Ad is attributable to its ability to infect a wide range of cell types, high efficiency of gene transfer, ability to incorporate large DNA inserts into its genome, and also efficient methods available for generation of recombinant viruses. In regard to the most commonly used Ad serotypes for gene delivery, Ad2 and Ad5 from subgroup C, binding of the globular knob domain of the Ad fiber protein with primary cellular receptor, coxsackievirus group B and adenovirus receptor (CAR), has been identified as the initial step of infection in in vitro studies\(^2\)-\(^4\). After administrated in mice, monkeys and pigs as a viral vector, wild type Ad or tropism unaltered recombinant Ad is mainly distributed in the liver, lung and spleen\(^5\)-\(^7\); however, the distribution is highly dependent on animal species and administration methods, e.g. intramuscularly, intravenously or intraperitoneally.

To fulfill gene delivery to CAR-deficient cell types and circumvent host neutralization of Ad vectors, modification on capsid proteins has been explored by genetically incorporating peptide ligands on fiber, penton base and hexon\(^8\)-\(^13\). However, major capsid proteins are crucial for Ad assembly and stability, and their structural properties only allow for incorporation of constrained heterologous ligands, which apparently have size limitation when added to the C terminus and HI loop of fiber\(^14\),\(^15\) and hexon L1 loop (Matthews et al., unpublished data).

pIX is expressed at delayed early stage with a molecular weight of 14.3kDa, and is a minor capsid protein associated with group-of-nine (GONs), acting as a stabilizing cement via its N-terminal domain interaction\(^16\)-\(^19\). pIX is considered not required for virus assembly\(^20\); however, it is essential for thermostability and packaging of full length
Recent Cryo-EM data suggested a theoretical possibility that the C terminus of pIX, which either binds on the capsid surface or extends outward from the capsid, can tolerate considerable modification and be utilized as an anchor for the addition of heterologous ligands to Ad particles. This thought was further demonstrated by several studies on Ad tropism alteration, in vivo imaging and tracking of physical Ad particles and cancer therapeutics via genetically incorporating corresponding heterologous polypeptides on pIX C terminus.

Therefore, pIX is an attractive candidate for the placement of functional motifs, and based on previous studies of pIX modification, we hypothesize that pIX may be utilized as a locale to accommodate multiple functional motifs on a single virion. To test this hypothesis, we attempted to incorporate three types of pIX into a single virion via genetic modification. We created a recombinant type 5 Ad carrying three types of genetically modified pIXs, each of which contained a distinct tag at its C terminus. Using Western Blotting analysis and immunogold labeling electron microscopy, we demonstrated that three types of pIX were incorporated into the virus and could coexist on a single virion. This is, to our knowledge, the first derived triple mosaic Ad, suggesting the possibility of further radical capsid design for simultaneously employing multiple functional modalities.

RESULTS

Generation of genetically pIX-modified Ad5 vectors. Since pIX has been shown to be an incorporation site of heterologous polypeptide ligands on human adenovirus (Ad) capsid without perturbation of viral viability, capsid stability or loss of peptide
functionality\textsuperscript{26, 29, 30, 33, 34}, it is possible that multiple pIXs carrying different ligands can be incorporated and presented in a single virion. To investigate this possibility, and test whether three types of pIXs can be efficiently incorporated into Ad, we constructed E1/E3-deleted Ad5 vector carrying three modified pIX genes containing Flag, hexahistidine (His\textsubscript{6}) or monomeric red fluorescent protein (mRFP1) sequences at their 3’ ends immediately before the stop codons. To minimize internal homologous recombination among modified pIX genes, we employed human cytomegalovirus (CMV) immediate-early promoter to drive IX-Flag gene expression and simian virus 40 (SV40) early promoter to drive IX-His\textsubscript{6} gene expression, respectively. These two pIX-expression cassettes were inserted upstream of the IX-mRFP1 gene driven by the native pIX promoter, resulting in an Ad plasmid carrying three modified pIX genes (Fig. 1B). Meanwhile an Ad plasmid containing only two pIX genes (IX-Flag and IX-mRFP1) was created for the purpose of comparison. These two Ad plasmids, pAd5-IXFlag-IXmRFP1 and pAd5-IXFlag-IXHis\textsubscript{6}-IXmRFP1, were transfected into 293 cells to generate pIX-modified viruses, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis\textsubscript{6}-IXmRFP1. These two viruses were successfully rescued and their genomic structures were verified by restriction enzyme digestion. An Ad5IXmRFP1 virus containing a single modification of C-terminal mRFP1 in pIX\textsuperscript{30} was used as a control and its construct was shown in Fig. 1B.

**Incorporation of modified pIXs in Ad capsid.** Western blotting analysis was used to confirm the incorporation of three heterologous pIXs into viral capsid. The CsCl-purified viral particles of Ad5-IXFlag-IXHis\textsubscript{6}-IXmRFP1 were denatured by boiling and the capsid proteins were dissolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Figure 1 Schema of Ad pIX modification. (A) Structural diagram of Ad5 vector with triple modifications. Flag, His$_6$ polypeptides and mRFP1 protein were incorporated in the C terminus of pIX. (B) Constructs of modified pIX genes in genomes of Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis$_6$-IXmRFP1. IX-Flag and IX-His$_6$ were driven by CMV and SV40 promoter, respectively, and inserted in Ad E1 region. “En” stands for SV40 enhancer. IX-mRFP1 was located at native region of pIX gene, driven by its native promoter (not shown here). (C) Amino acid residues of Flag and His$_6$ polypeptides and mRFP1 protein (incomplete) that were incorporated into the C terminus of pIX.
(SDS-PAGE). The probing of separated viral proteins of Ad5-IXFlag-IXHis6-IXmRFP1 using mouse anti-Flag, anti-His6 antibodies and a rabbit anti-pIX antibody detected the presence of protein bands with molecular weights of 15kDa, 21kDa, and 50KDa, corresponding to IX-His6, IX-Flag, and IX-mRFP1 fusion proteins, respectively (Fig. 2). The presence of IX-mRFP1 protein was further confirmed by fluorescence microscopy on intact viral particles. Purified virions were directly visualized under a fluorescence microscope to detect the red fluorescent signal of mRFP1 protein, and Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1, and Ad5-IXFlag-IXHis6-IXmRFP1 viruses were demonstrated to contain mRFP1 protein in viral capsid (Fig. 3). Of note, two mosaic viruses (Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis6-IXmRFP1 in panel C and D, respectively) had less fluorescent signal compared to single pIX-modified virus (Ad5IXmRFP1 in panel B), indicating that the incorporation levels of IX-mRFP1 fusion protein were lower in the two mosaic viruses.

**Presentation of modified pIX on virion capsid.**

To test whether or not the polypeptides incorporated into the C terminus of pIX were presented on viral surface and accessible to their specific antibodies, we performed enzyme-linked immunosorbent assays (ELISAs) in which mouse anti-Flag, anti-His6 antibodies and rabbit anti-RFP antibodies were used to detect three modified pIXs, respectively. The control Ad5 containing wild type pIXs was not recognized by any of these antibodies. The anti-Flag antibody recognized all three pIX-modified Ads since Flag tag was contained in IX-Flag and IX-mRFP1 fusion proteins. Ad5IXmRFP1 showed the highest level of interaction with anti-Flag antibody, and Ad5-IXFlag-IXmRFP1 had
Figure 2 Western blotting analysis of Ad vector containing triple pIX modifications. $10^{10}$ VPs of CsCl-purified Ad5-IXFlag-IXHis$_6$-IXmRFP1 were subjected to SDS-PAGE. The separated proteins were probed with rabbit anti-pIX (lane 1), mouse anti-His$_6$ (lane 2) or mouse anti-Flag (lane 3) antibody, and developed with AP-conjugated goat anti-mouse or anti-rabbit secondary antibodies. IX-mRFP1 contains a Flag peptide in the C terminus of pIX when originally constructed$^{30}$. The number on the left indicated the molecular mass in daltons. The (*) symbol was a non-specific band.
Figure 3 Direct visualization of pIX-modified Ad vector by fluorescence microscopy. 5×10⁹ VPs of CsCl-purified Ad were mounted on a microscope slide and examined under a fluorescence microscope with a ×100 objective using oil immersion. (a) Control Ad5 with wild type pIX; (b) Ad5IXmRFP1; (c) Ad5-IXFlag-IXmRFP1; (d) Ad5-IXFlag-IXHis₆-IXmRFP1. The thick arrows indicate heavily aggregated viral particles and the narrow arrows indicate slightly aggregated or single viral particles.
slightly weak interaction, while Ad5-IXFlag-IXHis<sub>6</sub>-IXmRFP1 had the lowest binding (Fig. 4A). The anti-His<sub>6</sub> antibody moderately bound to Ad5-IXFlag-IXHis<sub>6</sub>-IXmRFP1 (Fig. 4B). These results demonstrated that Flag, His<sub>6</sub> epitopes introduced into the pIX C termini were displayed on the surfaces of these pIX modified Ads and accessible for recognition by antibodies. However, anti-RFP antibody did not bind Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis<sub>6</sub>-IXmRFP1 as revealed by Fig. 4C, while efficiently bound to Ad5IXmRFP1. This could be due to that mRFP1 were masked by other epitopes (e.g. Flag or His<sub>6</sub>) in the heterotrimeric pIX protein, or the low incorporation rate of IX-mRFP1 proteins in Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis<sub>6</sub>-IXmRFP1, which was under the limit of the ELISA detection.

**CAR-dependent binding of pIX-modified adenovirus.**

Adenovirus has been shown to bind its primary cellular receptor CAR as the initial step of infection via its fiber knob protein. To examine whether the modification of pIX in Ad capsid interferes with the interaction, we performed the binding assay in 293 cells in the presence or absence of soluble CAR (sCAR) protein, which was used as competitive inhibitors. The presence of sCAR (200 µg/ml) blocked > 90% cell binding of Ad5, Ad5IXmRFP1 and Ad5-IXFlag-IXHis<sub>6</sub>-IXmRFP1, > 70% of Ad5-IXFlag-IXmRFP1 (Fig. 5A), indicating that the cell binding of all pIX modified Ad5 is still CAR-dependent.

**Infectivity of pIX-modified Ad5 vectors.**

Since capsid modification may affect the native infectivity of Ad5, we carried out infection assay in four CAR positive cell lines: 293, A549, GH329 and HeLa. Because
**Figure 4** Presentation of modified IX proteins on Ad capsid surface. (A) $10^9$ VPs of CsCl-purified Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1, and Ad5-IXFlag-IXHis$_6$-IXmRFP1 virus (designated as “Single”, “Double” or “Triple” in the figure, respectively) were subjected to a serial dilution (1, 2, 4, 8, 16, 32, 64, 128) and immobilized on an ELISA plate. Then the viruses were probed with mouse anti-Flag (A), mouse anti-His$_6$ (B) and rabbit anti-RFP (C) antibodies, which were further detected using alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit secondary antibody. Wild type Ad5 was used as a negative control. Each point represents the mean and standard deviation (SD) of triplicate determinations. Some error bars standing for SDs are smaller than their symbols.
Figure 5 Cell binding and infectivity of pIX-modified Ads. (A) CAR Binding inhibition assay. 293 cells were incubated with Ad5, Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis$_6$-IXmRFP1 (designated as “Single”, “Double” or “Triple” in the figure, respectively) at an MOI equivalent to 1000 VP/cell in the presence of 200 µg/ml sCAR protein at 4°C. After two hour, total DNA was extracted from cells and Ad5 genome copy numbers were determined by quantitative real-time PCR (Q-PCR). Values represent the relative amounts of Q-PCR readings and are expressed as percentage of cell binding relative to the corresponding control virus without adding sCAR protein (white bars). (B) pIX-modified Ad mediated genome transfer. 293, A549, GH329 and HeLa cells were infected with Ad5, Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis$_6$-IXmRFP1 at an MOI of 100 VP/cell at 37°C. Total DNA were extracted from virus infected cells after two hours, Ad genome copy numbers were determined by Q-PCR and plotted on the Y-coordinate. Each bar represents the mean and SD of triplicate determinations, and asterisks indicate significant difference of infectivity between pIX-modified Ads and control Ad5. The actual measurements in 293 and HeLa cells were showed above the bars.
there is no appropriate reporter gene in the pIX-modified Ads, we could not perform traditional infectivity assay based on transgene expression, e.g. luciferase activity. Nonetheless, we attempted to use the total viral genomes transferred into cells following 2-hour incubation as an assessment of infectivity. After infection, the cells were harvested by trypsin/EDTA and the total DNA were extracted for the measurement of total Ad5 genome copy number by quantitative real-time PCR (Q-PCR). All pIX-modified viruses exhibited similar infectivity and showed no significant difference among each other (Fig. 5B). In A549 and GH329 cells, pIX-modified viruses showed comparable infectivity with control Ad5; nevertheless in 293 and HeLa cells, infectivity of pIX-modified viruses was approximately one half of the control Ad5.

**Thermostability of pIX-modified Ads.**

pIX is a cement protein and stabilizes Ad capsid. This raised a question that whether the modification on pIX could affect the stability and structure integrity of the viral particle. Thus we compared the thermostabilities of pIX-modified Ads to that of control Ad5 with wild type pIX. CsCl-purified viruses were incubated at 45°C for various time, followed by re-titration using TCID\(_{50}\). All pIX-modified viruses exhibited compromised stability to some extent compared to control Ad5 when exposed at 45°C for various time (Fig. 6A and 6B). Specifically, after 40-min incubation, the remaining titer of Ad5IXmRFP1, which contains single pIX modification, was slightly reduced compared to control Ad5 when incubated in medium (P = 0.053), but severely reduced when incubated in PBS (P = 0.017). The remaining titer of Ad5-IXFlag-IXmRFP1, which contains double pIX modifications, was severely reduced when incubated in medium (P = 0.049) and in PBS.
(P = 0.016). The remaining titer of Ad5-IXFlag-IXHis₆-IXmRFP1, which contains triple pIX modifications, was slightly reduced when incubated in medium (P = 0.081) and in PBS (P = 0.13). The results shows that the thermostability of pIX modified Ad5 was compromised, which was consistent with an increased VP/PFU ratio observed in pIX modified viruses (98, 387, 391, and 245 for control Ad5, single, double, and triple pIX modified Ad5, respectively).

**Growth kinetics of pIX-modified Ad5.**

During the generation of Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis₆-IXmRFP1, we noticed that it took longer for transfected 293 cells to induce complete CPE compared with unmodified Ad5 (data not shown), indicating that the modification on pIX was likely to affect virus packaging. To obtain a quantitative understanding of this effect, growth kinetics of the pIX-modified viruses were studied and compared with that of wild type Ad5. Fig. 6C showed that wild type control Ad5 induced a full CPE and produced a maximum yield of viral progeny 3 days post infection; however, even though all pIX-modified Ads grew at a rate similar to that of control Ad5 in the first 3 days, they took 8 days to completely induce CPE with the yield of 2 log magnitudes lower than that of control Ad5 approximately. These data implied that the modification on pIX had an adverse effect on Ad’s packaging resulting in more defective viral particles in population, or the modification downregulated viral protein synthesis.
Figure 6 Thermostability and growth kinetics of pIX modified Ads. (A) Heat-inactivation of viral particles. CsCl-purified Ad5, Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis\textsubscript{6}-IXmRFP1 viruses (designated as “Single”, “Double” or “Triple” in the figure) were incubated in growth media or in PBS at 45°C. After various time points post infection, the viral infectious titers were re-determined by TCID\textsubscript{50} assay. Each point represents the mean and SD of duplicate determinations. (B) Growth Kinetics of Ads in 293 cells. 293 cells were infected with Ad5, Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis\textsubscript{6}-IXmRFP1 viruses at an MOI of 5 VP/cell. Cells were harvested with medium at different time points until full CPE was induced, and total viruses were determined by TCID\textsubscript{50} assay and plotted on the diagram as growth curves. Each point represents the mean and SD of duplicate determinations.
**Immunoelectron microscopy.**

Using Western blotting analysis on purified viral particles, we have demonstrated that three types of modified pIXs were incorporated into the Ad5-IXFlag-IXHis$_6$-IXmRFP1 capsids. However, it is possible that three types of pIXs were not incorporated into one single virion and those modified IX proteins came from different viral populations. To examine whether a single virion could display all three types of pIX, we performed immunogold electron microscopy to directly visualize pIX-modified Ads. Mouse anti-Flag, goat anti-His$_6$ and rabbit anti-RFP antibodies were used to detect IX-Flag, IX-His$_6$ and IX-mRFP1 proteins in viral capsids, followed by three secondary antibodies conjugated with 10, 18, and 25 nm gold particles, respectively. Control Ad5 displaying wild type pIX did not bind any antibody as shown in Fig. 7a. The Ads containing single pIX modification (Ad5IXFlag, Ad5IXHis$_6$ and Ad5IXmRFP1) were recognized by corresponding primary and secondary antibodies (Fig. 7, b-d). The Ad containing double pIX modifications (Ad5-IXFlag-IXmRFP1) was recognized by anti-Flag and anti-RFP antibodies, and labeled with 10 and 25 nm gold particles (Fig. 7e). The Ad containing triple pIX modifications (Ad5-IXFlag-IXHis$_6$-IXmRFP1) was recognized by anti-Flag, anti-His$_6$ and anti-RFP antibodies and labeled with 10, 18 and 25 nm gold particles as shown in Fig. 7f. The results showed that three types of modified pIXs could coexist on one single viral particle. Since a Flag tag is also present at the N terminus of mRFP1 protein, we cannot exclude the possibility that the all Flag epitopes detected in EM experiments came from IX-mRFP1 protein in the double and triple pIX-modified viruses. Nonetheless, this possibility is very rare because incorporation rate of IX-Flag protein
Figure 7 Immuno-gold electron Microscopy on pIX-modified Ads. Viruses were loaded onto EM grids, probed with gold nanoparticle conjugated antibodies, and observed under electron microscope at 60KV. (a) Ad5; (b) Ad5IXFlag; (c) Ad5IXHis\textsubscript{6}; (d) Ad5IXmRFP1; (e) Ad5-IXFlag-IXmRFP1 and (f) Ad5-IXFlag-IXHis\textsubscript{6}-IXmRFP1. Primary and gold-conjugated secondary antibodies employed were following: mouse anti-Flag, goat anti-His\textsubscript{6}, and rabbit anti-RFP primary antibodies were used for detecting 3 epitopes; 10 nm gold-donkey anti-mouse, 18 nm gold-donkey anti-goat, and 25 nm gold-donkey anti-rabbit secondary antibodies for gold labeling. Solid thin arrows point 10 nm gold particles, empty arrows point 18 nm gold particles and solid thick arrows point 25 nm gold particles. Scale bars in each panel represent 50 nm in length.
Figure 8 Generation of pIX mosaic Ad5 by co-infection. 293 cells were co-infected with pIX modified Ads (each virus carries one type of pIX) in the following combination at MOI of 20 VP/cell: a, Ad5IXFlag and Ad5IXHis\textsubscript{6}; b, Ad5IXFlag and Ad5IXmRFP1; c, Ad5IXHis\textsubscript{6} and Ad5IXmRFP1; d and e, Ad5IXFlag, Ad5IXHis\textsubscript{6} and Ad5IXmRFP1. CsCl-purified viral particles were stained with corresponding primary and gold-conjugated secondary antibodies as following: mouse anti-Flag, goat anti-His\textsubscript{6}, and rabbit anti-RFP primary antibodies were used for detecting 3 epitopes; 10 nm gold-donkey anti-mouse, 18 nm gold-donkey anti-goat, and 25 nm gold-donkey anti-rabbit secondary antibodies were used for gold labeling. Solid thin arrows point 10 nm gold particles, empty arrows point 18 nm gold particles and solid thick arrows point 25 nm gold particles. Scale bars in each panel represent 50 nm in length.
was much higher than that of IX-mRFP1 protein as shown in Western blotting of viral particles.

**Generation of triple mosaic Ad by co-infection.**

A co-infection strategy has been employed by Takayama *et al.* in generating mosaic Ad, in which two Ad genomes containing distinct serotypes of fiber were co-transferred into 293 cells. In this situation, both fibers were expressed in 293 cells and assembled into virion at random, resulting in fiber mosaic Ad. We therefore utilized this strategy as an alternative method to prove the principle of triple mosaicism on pIX. By co-infecting 293 cells with Ad5IXFlag, Ad5IXHis6 and Ad5IXmRFP1, each of which contains a single type of modified pIX, three IX-ligand fusion proteins were expressed for the production of viral progenies. The results showed that all kinds of double and triple pIX mosaic Ads were successfully generated by co-infecting 293 cells with Ad5IXFlag, Ad5IXHis6 and Ad5IXmRFP1 in corresponding combinations (Fig. 8), indicating that pIX mosaicism is a realizable feature of Ad and co-infection strategy is also an effective and efficient to generate pIX mosaic virus.

**DISCUSSION**

Adenovirus based viral vector has been utilized in a large number of gene therapy studies via a variety of modifications. Recent evidences suggested that incorporation of functional motifs into Ad capsid has several advantages over conventional gene therapy strategies which express transgenes after infection. For examples, with regard to viral vector imaging, the expression of reporter genes can only provide indirect information of
virus localization and replication, which is determined by transcriptional and translational activity of that particular transgene in the target cells, and possibly cause nonspecific expression in non-target cells. However, if linked on Ad capsid, polypeptides function directly after viral infection with no need of expression. This property is especially important for transducing certain cell types where promoter activity is low. This thought was explored in the study of monitoring oncolytic adenoviruses in vivo$^{30}$, where the physical trafficking and distribution of Ad were under observation with an imaging modality incorporated on pIX. The C terminus of pIX was suggested to be a potential local to incorporate functional modalities in several studies. Besides the imaging motif$^{30}$, thymidine kinase can also be incorporated in this locale with its native function maintained$^{32}$, suggesting Ad vector’s potentials in oncolysis and cancer therapy. Previous studies$^{36,37}$ suggested that transductional targeting via incorporating targeting ligand on the C terminus of pIX usually is not as effective as doing thus on fiber knob, partially due to its relatively low accessibility by virtue of the topology and dimensional arrangement of pIX proteins within GONs and steric hindrance effect from fibers. This issue may be circumvented by lifting the targeting ligand toward the hexon top via alpha-helical spacers to improve accessibility as illustrated by Vellinga et al.$^{34}$, although this hypothesis is under debate$^{36,37}$. In addition, better understanding of organ and tissue’s physical barriers makes it possible to deliver viral vectors in vivo more efficiently than before by utilizing various transcytosis machineries$^{38-42}$, which needs an extra relaying motif for transcytosis besides the targeting ligand against the receptor of destination. On the other hand, development of viral vectors may also be favored in terms of efficacy and reliability by therapeutic synergism and imaging redundancy, both of which need more
than one incorporation site to accommodate the synergistically therapeutic motifs or an extra imaging motif on the viral capsid.

In this proof-of-principle study, we explored the possibility of incorporating three different pIX proteins into a single Ad5 virion capsid by replacing the native pIX gene with three expression cassettes encoding three modified pIX proteins containing Flag, His$_6$ polypeptides or mRFP1 protein at the C terminus, respectively. The Ad containing double or triple pIX modifications, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis$_6$-IXmRFP1, were successfully rescued. The three modified pIXs were incorporated into viral capsid and accessible to antibodies. By labeling pIX modified viruses with three tag-specific antibodies which were conjugated with gold nanoparticles in three different sizes, we have showed that two or three heterologous IX proteins could coexist on a single virion of Ad5-IXFlag-IXmRFP1 or Ad5-IXFlag-IXHis$_6$-IXmRFP1, respectively.

However, pIX-modified Ads appeared be more thermolabile than control Ad5 as shown in the heat inactivation assay (Fig. 6A and 6B), which is not unexpected since pIX plays an important role in Ad stability. Of note, Ad5-IXFlag-IXHis$_6$-IXmRFP1 seems to be more thermostable than AdIXmRFP1 and Ad-IXFlag-IXmRFP1. This can be explained by that the capsid of triple pIX modified viruses contains more IX-Flag and IX-His$_6$ proteins, which are small in size and do not affect the cement function of pIX and overall stability of Ad5 virion$^{26}$. In the growth kinetics experiment (Fig. 6C), the functional multiplicity of control Ad5, Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1, Ad5-IXFlag-IXHis$_6$-IXmRFP1 were 0.050, 0.013, 0.013, 0.020 PFU/cell, respectively. Thus, the 2-log difference of recovered viral progenies after 24 hours between control Ad5 and pIX modified virus could hardly be explained by different initial infection. This, together
with the delayed full CPE formation in pIX modified viruses, suggested that modification on pIX had adverse effect on viral replication and/or assembly. These data was consistent with previous studies, in which deletion, mutation and modification of pIX was suggested to perturb Ad’s stability\textsuperscript{18-21, 26}. In particular, pIX whole protein was essential for genome packaging and stability, and residues 13-15 and 22-28 at the N terminus were vital since deletion of these residues made viruses seriously thermolabile. The central region and C-terminal domains seemed to be trivial in this regard because point mutations (L114P and V117D) and deletions on residues 60-72 (alanine stretch)/100-114 (large part of leucine repeat) neither caused problems in pIX incorporation nor viral thermolability, arguing for a good tolerance of modification in these two regions. However, the properties of heterologous peptide \textit{per se}, such as structure, size and electric charge, may affect the stability\textsuperscript{26}. In addition, pIX was suggested to be involved in virus-induced nuclear reorganization and inhibiting cellular antiviral responses\textsuperscript{18, 43}, and act as a transcriptional activator\textsuperscript{44}, although the significance is under controversy\textsuperscript{45}. Therefore, structural compromise on leucine repeat at the C terminus and alanine stretch at the central region of pIX may also be a cause of the delayed CPE induction by pIX-modified Ads.

Three heterologous pIXs were incorporated into viral capsid as revealed by western blotting analysis on lysed viral protein as well as fluorescence microscopy, ELISAs, and immunogold EM on purified viral particles. Nevertheless, these data also suggested that three types of pIX were not equally incorporated. In Western blotting analysis, IX-Flag protein appeared to be the most and IX-mRFP1 seemed to be the least. Fluorescence microscopy, ELISA and immunogold EM also suggested possible low incorporation rate of IX-mRFP1 protein. The unequivocal stoichiometry of IX proteins
could be by virtue of their synthesis since three modified pIX genes were driven by three different promoters (CMV, SV40, and pIX native promoter). These three promoters apparently had different activity since protein expression of modified pIXs in Ad infected cells were quite different both in time and strength (data not shown). In addition, the properties of modified pIX such as size, charge and conformation could also cause aggregation and incompatibility, and interfere with viral assembly (Ugai et al., unpublished data).

By labeling the three types of pIXs with gold nanoparticles in three different sizes, EM provided a direct evidence of the coexistence of heterologous IX-ligand fusion proteins in viral capsid, and further demonstrated that the triple mosaicism could be the feature of a single virion rather than mixed viral populations. It is noteworthy that, although there are 240 copies of pIX molecules in each viral particle, only a few gold nanoparticles were bound on Ad5-IXFlag-IXmRFP1 or Ad5-IXFlag-IXHis6-IXmRFP1 virus. This is probably due to spatial hindrance effect of gold particles, and we chose big gold particles for easier differentiation, which have relatively high spatial hindrance effect against each other during staining. Besides, the low efficiency of triple staining may be the reason of the scattered staining pattern shown in the figure. Therefore, it is not unexpected that the frequency of triply-labeled Ads is low that only about 2% viral particles were positively stained with all three gold particles. Taken together, we have demonstrated for the first time a triple mosaic capsid configuration based on pIX modification, and validated the possibility of further radical engineering in pIX.

MATERIALS AND METHODS
**Antibodies.** The pIX-specific antibody was a kind gift from Dr. I. Dmitriev (Gene Therapy Center, University of Alabama at Birmingham). The mouse anti-His$_6$ monoclonal antibody was purchased from Qiagen (Valencia, CA.). The goat polyclonal anti-His$_6$ antibody was purchased from Abcam (Cambridge, MA.). The anti-Flag M2 monoclonal antibody was purchased from Sigma (St. Louis, MO.). The rabbit polyclonal anti-RFP antibody was purchased from Chemicon (Temecula, CA.). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies, or alkaline phosphatase (AP)-conjugated goat anti-mouse, goat anti-rabbit secondary antibodies, and electron microscopy (EM) grade 18 nm colloidal gold-conjugated donkey anti-goat were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA.). EM grade 10 nm gold-conjugated donkey anti-mouse, EM grade 25 nm gold-conjugated donkey anti-rabbit secondary antibodies were purchased from Electron Microscopy Science (EMS, Ft. Washington, PA.).

**Cells.** The human embryonic kidney cell line (293) transformed with Ad5-E1 DNA, human lung carcinoma (A549) cells and human cervix epithelial carcinoma cells (HeLa and GH329) were purchased from American Type Culture Collection (ATCC, Manassas, VA.). All cell lines were cultured in Dulbecco’s modified Eagle medium-Ham’s F12 (50/50) medium (Sigma) containing 10% fetal calf serum (HighClone, Logan, Utah), 2 mM L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and grown at 37°C in a 5% CO$_2$ humidified incubator.

**Construction of recombinant plasmids.**
The E1/E3 deleted Ad5 genome containing three modified pIX genes were constructed as following sequentially:

(i) Generation of shuttle vector carrying two modified pIX genes, pShuttle-IXFlag-IXmRFP1. The IX-Flag open reading frame (ORF) was obtained by PCR on the template of a IX-Flag containing shuttle plasmid named pShlpIXFlagNheI\textsuperscript{26}, using primers KpnI (s), \textit{5'- GC GGTACC ATGAGCACCAACTCGTTTGATGGAAGC - 3'}, and HindIII (as), \textit{5'- CCC AAGCTT CTACCGCGCGCAAAACCCCTAA - 3'}, with corresponding recognition sequence underlined. Then the PCR product was digested and cloned into pShuttle-CMV plasmid (Stratagene), resulting in pShuttle-CMV-IX-Flag-pA. The expression cassette CMV-IX-Flag-pA was amplified by PCR with primers NotI (5’), \textit{5'- GCAAGGGT GCGGCCGC TAATAGTAATCAATTACGGGGTCAT TAG - 3'}, and SalI (3’), \textit{5'- GGCGC GTCGAC TAAGATACATTGAGTTGGGTGCTAC - 3'}, and subcloned into pShuttle-IX-mRFP1\textsuperscript{30}, resulting in a shuttle vector containing two modified pIX genes: pShuttle-IXFlag-IXmRFP1.

(ii) Generation of the final shuttle vector containing three modified pIX genes, pShuttle-IXFlag-IXHis\textsubscript{6}-IXmRFP1. To incorporate sequence encoding six consecutive histidines into the 3’ end of the pIX gene (pIX-His\textsubscript{6}), two primers HindIII (5’), \textit{5'- CCC AAGCTT ATGAGCACCAACTCGTTTGATGGAAGC - 3'}, and XbaI-6H (3’), \textit{5'- GC TCTAGA TTA GTG ATG GTG ATG ATG AACCGCATT GGGAGGGGAGGAAGC - 3'}, were used with corresponding recognition sites, stop codon and the sequence underlined, respectively. The resultant PCR product was cloned into pGL3-Control shuttle vector (Promega Corporation, Madison, WI) containing simian virus 40 (SV40) early promoter using two-enzyme digestion (HindIII and XbaI). The expression cassette SV40-IX-His\textsubscript{6}-
pA in pGL3-IX-\textit{His}_6 was excised by two-enzyme digestion (SalI and XhoI), and cloned into SalI digested pShuttle-IXFlag-IXmRFP1, resulting in final shuttle vector containing 3 modified pIX genes, pShuttle-IXFlag-IX\textit{His}_6-IXmRFP1.

(iii) Generation of pIX-modified adenoviral genomes by homologous recombination in \textit{Escherichia coli}\textsuperscript{46}. The shuttle vector pShuttle-IXFlag-IXmRFP1 or pShuttle-IXFlag-IX\textit{His}_6-IXmRFP1 was linearized with PmeI restriction enzyme and homologously recombined with pAdEasy-1 (Stratagene, La Jolla, CA.) in electrocompetent BJ5183-Ad1 (Stratagene). The generated adenoviral genomes contain two (IXFlag-IXmRFP1) or three (IXFlag-IX\textit{His}_6-IXmRFP1) modified pIX genes in E1 region. The constructs of resultant Ad plasmids pAd5-IXFlag-IXmRFP1 and pAd5-IXFlag-IX\textit{His}_6-IXmRFP1 were confirmed by restriction digestions and sequencing.

\textbf{Virus rescue, propagation and purification.}

pAd5-IXFlag-IXmRFP1 and pAd5-IXFlag-IX\textit{His}_6-IXmRFP1 were linearized with PacI restriction enzyme; then the large fragments (32.3 kb and 33.4 kb, respectively) were purified and transfected into 293 cells grown in 25 cm\textsuperscript{2} flasks with Superfect (Qiagen). The cells were collected when evident cytopathic effect (CPE) were observed, and disrupted by four freeze and thaw cycles. The lysates were centrifuged at 3,000×g for 5 min at 4°C to move the cell debris. The released viruses in the supernatant were subsequently used for further propagation until sufficient 293 cells were infected (> ten 175 cm\textsuperscript{2} flasks). The Ad in infected cells were purified essentially as described previously\textsuperscript{13}. In brief, the cells were lysed by four freeze and thaw cycles and centrifuged at 3,800×g for 30 min at 4°C to move the cell debris. The cell extracts containing the
viruses were loaded on the top of a 1.33/1.45 CsCl step gradient and centrifuged at 55,000×g for 3 hours at 4°C. Lower band containing infectious particles were re-centrifuged on another 1.33/1.45 CsCl step gradient at 100,000×g for overnight at 4°C. The resulting band of adenoviruses was collected and dialyzed four times against 500 ml phosphate buffered saline (PBS) containing 10% glycerol, 2 hours each time. The generated Ads were designated as Ad5-IXFlag-IXmRFP1 (containing two types of pIX) and Ad5-IXFlag-IXHis6-IXmRFP1 (containing three types of pIX). Viral particle titers were determined by spectrophotometry at OD260.

**Protein electrophoresis and Western blotting.**

Purified viruses were boiled in Laemmli sample buffer for 5 min and separated on 4 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked in 5% milk in Tris-buffered saline containing 0.05% Tween-20 followed by primary antibodies incubation (rabbit anti-pIX, 1:1000; mouse anti-Flag, 1:1000; mouse anti-His6, 1:1000; rabbit anti-RFP, 1:1000). After washing and re-blocking, the membrane was incubated with corresponding secondary antibodies conjugated to HRP or AP at 1:1000 dilution. The HRP signal was developed with ECL plus Western blotting detection system (GE healthcare, Little Chalfont, UK), detected with BioMax MR scientific imaging film (Kodak, Chalon-sur-Saone, France) and medical film processor SRX-101A (Konica, Tokyo, Japan). AP signal was developed with AP staining solution containing 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitro blue tetrazolium (NBT) according to the manufacturer protocol (Sigma).
ELISA.

Solid-phase binding enzyme-linked immunosorbent assays (ELISAs) were performed essentially as described previously. Briefly, $10^9$ VP viruses were subjected to serial dilution (1, 2, 4, 8, 16, 32, 64, 128) in 100 µl of 100 mM carbonate buffer (pH 9.5), and immobilized triplicately in a 96-well plate (Nunc Maxisorp) by overnight incubation in at 4°C. After 4 washes with 0.05% Tween 20 in Tris-buffered saline (TBS-T) and blocking with TBS-T containing 2% bovine serum albumin (BSA), the viruses were probed with primary antibody, and then AP-conjugated secondary antibodies in TBS-T containing 0.5% BSA at room temperature for 2 hours, with extensive washes and blocking in between. $p$-nitrophenyl phosphate (Sigma) was used for color development as described by the manufacturer, and light absorbance (405 nm) was obtained by a microplate reader (PowerWave HT 340, BioTek, Winooski, VT.) after incubation for 90 min.

Fluorescence microscopy.

Fluorescence microscopy on purified viral particles was performed as following. 10 µl 50% glycerol/PBS solution containing $5 \times 10^9$ VPs of viruses were dropped onto a Fisherfinest® premium microscope slides (Fisher Scientific, Pittsburgh, PA.) and covered by Fisherband® #1 cover glass (Fisher Scientific). Fluorescence microscopy was performed with an inverted IX-70 microscope (Olympus, Melville, NY) equipped with a Magnifire digital CCD camera (Optronics, Goleta, CA.).
Quantitative polymerase chain reaction (Q-PCR).

For quantification of adenoviral E4 DNA, TaqMan primers and probes were designed by the Primer Express 1.5 software and synthesized by Sigma Genosys (Woodlands, Texas). The sequences of primers and probe to amplify E4 gene were following: forward primer 5’ - GGAGTGCGCGCCGAGACAAAC - 3’, reverse primer 5’ - ACTACGTCGCGCGTTCCAT - 3’ and probe 6FAM-TGGCATGACACTACGACCAACACGATCT - TAMRA. With optimized concentration of primers and probe, the components of Real-Time PCR mixture were designed to result in a master mix with a final volume of 9 µl per reaction containing 1X Universal PCR Master Mix (Applied Biosystems, Foster City, CA.), 100 nM forward primer, 100 nM reverse primer, 100 nM probe and 0.025% BSA. For the assay, known amount of E4 template DNA (10^8, 10^6, 10^4 and 10^2 copies/µl) was amplified to generate a standard curve for quantification of the E4 copy numbers of samples. 1 µl of sample was added to 9 µl of PCR mixture in each reaction capillary. Two no-template-control capillaries received 1 µl of water. All capillaries were then sealed and centrifuged using LC Carousel centrifuge (Roche Molecular Biochemicals, Indianapolis, IN.) to facilitate mixing. PCR was carried out using a LightCycler™ system (Roche Molecular Biochemicals). Thermal cycling conditions were following: 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. Data was analyzed with LightCycler software 3.

CAR binding inhibition assay.
293 cells were plated in 48-well plates at a density of \(6 \times 10^4\) cells per well the day before binding, on which viral particles equivalent to an MOI of 1000 were used for binding assay. Virus were pre-incubated with sCAR (200 \(\mu\)g/ml) protein in DMEM/F12 medium containing 2% FBS at room temperature for 10 min, and then were incubated with cells at 4°C. After 2 hours, cells were washed with cold PBS, harvested by Versene/EDTA. Total DNA was extracted from collected cells using QIAamp DNA Blood mini kit (Qiagen) and subjected to Q-PCR to measure Ad5 E4 copy numbers.

**Genome transfer assay.**

293, A549, GH329 or HeLa cells were plated in 12-well plates at a density of \(3 \times 10^5\) cells per well the day before infection. Viral particles equivalent to an MOI of 100 were used for each infection in DMEM/F12 medium containing 2% FBS at 37°C. After two hours, the infected cells were washed by PBS and harvested by Trypsin/EDTA. Total DNA was extracted from collected cells by using QIAamp DNA Blood mini kit (Qiagen) and subjected to Q-PCR to measure Ad5 E4 copy numbers.

**Heat inactivation assay.**

Heat inactivation assay was performed essentially as described previously\(^{13, 26, 48}\). Briefly, viruses were incubated at 45°C for 0, 5, 10, 20 or 40 min in either PBS (without Ca\(^{2+}\) and Mg\(^{2+}\)) or growth medium containing 2% FBS. Then their infectious titers were re-determined by standard Tissue Culture Infectious Dose 50 (TCID\(_{50}\)) method (AdEasy vector system, Qbiogene, Carlsbad, CA). In brief, 100 \(\mu\)l growth medium containing 2% FBS and \(10^4\) 293 cells were added on two 96-well flat bottom plates the day before
infection. Eight serial dilutions of the virus ranging from 10^{-3} to 10^{-10} were made in medium containing 2% FBS, and 100 µl of diluted viruses were added into 96-well plates, one row for each dilution. After incubation for 10 days at 37°C in 5% CO_2 humidified incubator, the plates were examined for CPE under microscope. Observable CPE containing wells were counted for each row in order to determine the ratio of positive wells per row in the 96-well plates. Titer was calculated by using KARBER statistical method: \( T (\text{TCID}_{50} \text{ titer}) = 10 \times 10^{1+d(S-0.5)} / \text{ml} \), in which \( d \) is the log 10 of the dilution and \( S \) is the sum of ratios from the first dilution.

**Statistical analysis.**

Statistical analysis was performed with two-tailed unpaired Student's \( t \)-tests among groups. P values <0.05 were considered statistically significant.

**Growth Kinetics.**

Growth kinetics of adenovirus was obtained essentially as described previously^{13}. 293 cells were plated in 6-well plates at the density of 3×10^5 cells per well 24 hours before infection. The cells were infected with adenoviruses at an MOI of 5 VPs/cell in 500 µl growth medium containing 2% FBS. 1.5 ml more growth medium containing 10% FBS was then added into each well after two hours incubation at 37°C in 5% CO_2 humidified incubator. The infected cells were monitored and harvested with medium at various time point post infection until complete CPE was formed. The collected cells together with the medium were lysed by four cycles of freeze-thaw in dry ice and water bath, and were subjected to centrifugation at 3000×g for 30 min at 4°C for cell debris removal. The total
viruses in each well were determined by multiplying TCID\textsubscript{50} titer with the total volume of the supernatant, and plotted on the diagram as growth curves.

**Immunoelectron microscopy.**

Viruses were fixed with 2% paraformaldehyde/PBS at room temperature for 10 min and adhered to 400-mesh nickel grids supported with carbon-coated Formvar film (EMS). After washing with 1%BSA/PBS twice for 10 min each, grids were probed with 1% BSA/PBS diluted primary antibodies (1:200 for goat anti-His\textsubscript{6}, M2 anti-Flag and rabbit anti-RFP) and incubated at room temperature for 1 hour. After 2 cycles of 1%BSA/PBS washes, grids were incubated with 1:40 diluted secondary antibodies (10 nm gold-donkey anti-mouse, 18 nm gold-donkey anti-goat, and 25 nm gold-donkey anti-rabbit) at room temperature for 30 min. After fixing with 1% glutaraldehyde/PBS for 20 min, grids were subjected to negative staining in 2% uranyl acetate for 12 seconds and examined under transmission electron microscope in UAB High Resolution Imaging Facility.

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DISCUSSION

The extensive work on the biology and pathology of the adenovirus has made it ideal for vector design. For this reason the adenovirus has become a valuable tool in the design of rational targeted cancer gene therapy and virotherapy strategies. As the first gene therapy product going beyond the stage of clinical trials, a replication incompetent adenoviral vector carrying the p53 gene (Gendicine, SiBiono GeneTech Co.) showed efficacy in shrinking head and neck tumors after intratumoral administration\textsuperscript{283}. There are several advantages for utilizing adenovirus as a gene delivery tool. First, with well evolved biological properties, adenovirus is stable \textit{in vivo} and can infect a wide range of cell types (both dividing and non-dividing) at a high efficiency, and can mediate the expression of transgenes at a high level. Second, recombinant adenovirus can accommodate large DNA inserts (up to 30kb in the case of gutless adenovirus), providing considerable flexibility when choosing transgene candidates. More importantly, the well established viral production protocols and an extensive collection of viral mutants provide a unique capability to study and create novel adenoviruses to fulfill the requirements of various gene therapy scenarios, including the treatment of brain tumors.

\textbf{Targeting Adenoviral Vectors to Brain Tumors}

Brain tumor research is a major focus due to the high incidence and high mortality rate associated with the disease. Brain tumors occur in people of all ages with an incidence rate of 14 per 100,000 persons. It is the most common of the solid tumors in
children and second leading cause of cancer-related deaths in children and adolescent under the age of 40\textsuperscript{284, 285}. Brain tumors represent a heterogeneous group of primary or metastatic intracranial neoplasms, each of which has its own biology, treatment, and prognosis\textsuperscript{286}. Among adults, the most frequent types of brain tumors are glioma as well as other astrocytic tumors, meningiomas, acoustic neuromas, and pituitary gland tumors (National Cancer Institute brain tumor study in adults: fact sheet, http://www.cancer.gov/cancertopics/factsheet/braintumorstudy).

Although these tumors remain confined to the brain, they have proven resistant to surgical, drug and radiation therapies due to a number of reasons: the invasive nature of the tumor within the brain, the substantial fraction of tumor cells in temporary growth arrest, the genotypic heterogeneity of tumor cells, the difficulty in delivery of drugs from the blood stream into the brain, and the poor immune surveillance in the CNS.

New therapeutic approaches to brain tumor are under investigation such as telomerase inhibitor\textsuperscript{287}, immunotherapy and oligonucleotide interfering therapy\textsuperscript{288-294}, antiangiogenic therapy\textsuperscript{295}, and gene therapy\textsuperscript{296}. However, surgery, radiation therapy, and chemotherapy remain the standard strategies to treat brain tumors. Despite the wide usage and recent progress in traditional cancer therapy measures, severe side effects, undesired toxicities, and inefficiency are common depending on the type, stage, and location of the tumor, especially in late-stage, metastatic tumors\textsuperscript{286, 297, 298}.

It has been recognized that the toxicity and inefficiency of cancer therapies are not independent events. Many chemotherapy agents (e.g. cyclophosphamide (Cytoxan)) are uptaken by normal cells and tissues after systemic administration, which results in a
wide spectrum of clinical toxicities exemplified by haemorrhagic cystitis (bladder damage), undesired immunosuppression, and alopecia (hair loss)\textsuperscript{299-301}.

Great interest and effort have been directed toward the development of targeted cancer therapeutics based on antibodies and small molecules directed at cancer specific targets such as CD20, HER-2/neu, EGFR, VEGF, PSMA, estrogen receptor, BCR/ABL, c-KIT, and FLT-3 (see the review\textsuperscript{302}). A typical example of success of targeted cancer therapy is the development and application of imatinib mesylate (Gleevec) in BCR/ABL translocation positive chronic myeloid leukemia (CML). Imatinib mesylate is a specific, adenosine triphosphate-binding inhibitor of BCR/ABL oncoprotein, c-KIT, and platelet-derived growth factor receptor (PDGF-R)\textsuperscript{303}, and potently inhibits protein tyrosine phosphorylation and anti-apoptosis activities in the CML tumor cells\textsuperscript{304, 305}. Treatment with imatinib can induce complete hematologic and cytogenetic remissions with only minimal toxicities in up to 80\% of patients, and has emerged as standard of care for early stage CML\textsuperscript{302, 306}. These results strongly suggest the advantages and benefits of targeted molecular medicine in cancer therapy, and indicate a direction for developing future therapeutic approaches to various diseases.

The plasma membrane of the cell acts as the most fundamental barrier and the major filtering system that restricts the uptake of bioactive molecules. This is also true for cancer therapeutics based on adenoviral vectors. The requirement of adenovirus native receptor CAR on cell membranes for the efficient cell entry and transduction of Ad viral vectors greatly limits the usage of Ad in cells that are deficient in CAR expression. Unfortunately many primary tumor cells express low levels of CAR\textsuperscript{307-318}, which has been demonstrated to be associated with low Ad transduction\textsuperscript{319}. Therefore, targeting Ad
to alternative cell entry pathways is necessary to fulfill efficient gene delivery into tumor cells.

The targeting effect of cancer therapeutics based on radical design of adenovirus could be achieved at transductional\textsuperscript{47}, transcriptional\textsuperscript{320-322}, and translational\textsuperscript{323, 324} levels. Restricting virus infection at the level of cell entry is definitively among the prime issues. Transductional targeting of adenoviral vectors is focused on the manipulation of viral entry pathways to achieve restricted or expanded tropism to certain cell types. For instance, adenoviral fiber protein, which is responsible for binding the adenovirus cellular receptor CAR as the initial step of cell entry, was genetically modified with an integrin-binding RGD motif, and allowed such derived viral vectors to transduce CAR-negative ovarian tumor cells at much higher efficiency compared with fiber-unmodified adenovirus\textsuperscript{186}. Therefore, besides increasing safety, transductional targeting can also make therapeutic viruses more efficient in gene delivery by diminishing the loss of virus to non-target cells. For transcriptional targeting, for example, a series of conditionally replicative adenoviruses (CRAds) have been developed and tested in clinical trials, and exhibit selective cytotoxicity to tumor cells with various mechanisms\textsuperscript{325-330}. For example, the ONYX-015 adenovirus\textsuperscript{331} was designed to replicate only in p53-deficient cells (the majority of human tumor cells\textsuperscript{332, 333}) because of the deletion in E1B 55K gene which inactivates p53 to prevent cell cycle arrest and apoptosis induction and is essential for adenoviral replication in normal cells. Translational control of therapeutic genes is also a potent measure to achieve targeting effects in tumors. In an adenovirus mediated suicide gene therapy, the cDNA of HSV-TK was modified by adding a modified long 5'-UTR to
achieve restricted translation in cells with elevated expression of an translation initiation factor eIF4F, as is the case in most human breast tumor cells\textsuperscript{334-338}.

A variety of cell-penetrating peptides (CPPs) have been suggested as good candidates for targeting ligand. CPPs conjugated to therapeutic agents have been shown to efficiently mediate cell uptake of such agents. As the most extensively studied CPP, PTDtat has shown its ability \textit{in vitro} and \textit{in vivo} to deliver small-molecule drugs such as polypeptides, proteins, DNA, and RNA into almost all mammalian cell types\textsuperscript{212}. This property is very important for tumor targeting since the tumor compartment is a mixture of highly diverse cell populations, where more than 50\% cells are cancer-associated fibroblasts, tumor endothelial cells and tumor-associated macrophages\textsuperscript{339}. For cancer therapy, PTDtat was usually coupled to the N termini of various tumor suppressor proteins, cell cycle regulators, apoptotic proteins, oncogenic and metastasis pathway inhibitors, immunogenic antigens, and achieved high transduction efficiency and significant therapeutic effects\textsuperscript{217}.

In our study presented in the chapter 1, we sought to employ PTDtat peptide to improve the gene transfer efficacy of Ad5 vectors by genetic modification of the fiber knob domain with a PTDtat motif. Our data demonstrated the success of this strategy. The fiber modified Ad5 vector, Ad5.PTDtat, not only exhibited enhanced gene delivery efficiency of Ad5 vectors in low-CAR cells that are resistant to unmodified Ad5 infection, but also in high-CAR cells that are permissive to Ad5 infection. The enhanced infectivity of Ad5.PTDtat was found to be mediated by targeting of PTDtat to negatively charged epitopes such as heparan sulfate containing proteoglycans on cell surface. In addition, we found PTDtat mediated Ad5.PTDtat infection is additive to native CAR-mediated
infection as assessed by competitive inhibition assays, which was not unexpected since Ad5.PTDtat maintained full CAR-binding activity. More significantly, the enhanced gene delivery efficacy of Ad5.PTDtat was demonstrated in vivo using low-CAR U118MG tumor models, and employment of a recently developed non-invasive optical imaging system, allowed us to visually detect the enhanced gene delivery in vivo.

Employment of PTDs to facilitate virus infection has been investigated previously, but only using non-genetic methods. In particular, chemically synthesized PTDs or bi-specific adaptor proteins consisting of PTDs and the extracellular domain of CAR have been used to arm Ad vectors, and resulted in enhanced gene delivery. Compared to the non-genetic methods, our genetically modified PTDtat vector has major advantages for two major reasons: 1) genetic modification allows stable interaction between Ad5 and the PTDtat targeting epitope, thus reducing the volatility associated with the affinity and stability of protein-protein interactions in the presence of different environmental factors. This is critical especially for in vivo applications and, 2) genetic modification does not require production of peptides or fusion proteins other than the viral vector, while large amounts of high quality protein/peptide production is required for nongenetic strategies (in addition to high quality production of the viral vectors), which is especially important for in vivo studies.

One issue associated with PTDtat-mediated protein delivery is the inefficient release of some PTDtat fusion proteins from the endosomal compartments. It has been demonstrated that a large proportion of the PTDtat fusion protein remains trapped in non-cytosolic compartments even though it is efficiently taken up by the cells, and this apparently would compromise the gene transduction and the therapeutic effects of the
modified viral vectors. In our study, we examined the distribution of Ad5.PTDtat particles in cells at various time points (from 0.5 hour to 4 hours) following addition of the viruses to the cells by immunofluorescent staining, and found that the distribution of Ad5.PTDtat inside the cells was similar to that of unmodified Ad5 vectors. This indicates endosomal trapping is not significant, if present at all, with Ad5.PTDtat infection of cells. This also suggests that the modification on fiber does not affect the fibers’ release from viral particles and the fiber’s role in regulating endosome escaping of adenovirus. In addition, the enhanced gene delivery mediated by Ad5.PTDtat confirmed that the virions were able to efficiently escape the endosomal compartment.

The potential utility of the infectivity-enhanced Ad5.PTDtat vector in cancer gene therapy was initially investigated in this study using low-CAR expressing tumor models. Indeed, many tumor cells have been shown to express very low levels of CAR, which is partially responsible for the low efficacy of Ad5 mediated cancer gene therapy in \textit{in vivo} studies, especially in clinical trials\textsuperscript{47, 346, 347}. As we mentioned earlier, the ability of Ad5.PTDtat to improve the gene delivery efficacy is attributable to the PTDtat motif, which binds to the negatively charged motifs expressed on cell surface, in particular, heparan sulfate containing proteoglycans that are widely expressed in a variety of cells including tumor cells\textsuperscript{348-350}. In addition to cancer gene therapy, Ad5.PTDtat may also be applied in other gene therapy applications where infectivity-enhancement is beneficial. Infectivity enhanced vectors will not only allow efficient gene delivery into low-CAR target cells, but also allow use of a reduced amount of viral vectors, thus reducing vector associated toxicity. Previous studies have developed several other infectivity enhanced vectors, which include Ad5 vectors modified with RGD, polylysine, or knobs from other
Ad serotypes\textsuperscript{186, 193, 351, 352}. Since each of the modified vectors uses a unique extra targeting motif, the enhanced gene delivery efficacy in a specific cell type depends on the expression of individual receptors on its cell surface. Similar to PTDtat, the polylysine epitope, which is composed of a stretch of lysine residues, is highly basic, and can utilize heparan sulfate as its receptor. Nonetheless, the interaction between PTDtat and heparan sulfate is not only based on ionic interactions, but also on the specific structures of the peptide and the proteoglycans\textsuperscript{232, 235, 353}. Therefore, the choice of an infectivity-enhanced vector needs to be determined for each specific application involving gene delivery enhancement. Indeed, given the fact that PTDs selectively interact with distinct glycosaminoglycan species and patterns\textsuperscript{354}, specific tumor or tumor subset targeting Ad vector could be design based on our strategy.

We herein presented the genetically modified Ad5.PTDtat vector. It maintains the ability to interact with Ad’s native receptor CAR so that it can deliver transgenes into both high-CAR and low-CAR cells more efficiently than the unmodified Ad5 vector. Our data further showed that Ad5.PTDtat infected cells via both CAR and PTDtat pathways. More significantly, Ad5.PTDtat exhibited enhanced gene delivery \textit{in vivo} in a tumor model, and thus may be useful for gene therapy applications involving low gene delivery efficacy.

Of note, the tumor transduction efficiency of the Ad5.PTDtat could be further enhanced by strengthening the transcellular property of PTDtat when combined with the HSV-TK/GCV therapy that we will mention in the last part of this discussion. The cytotoxic mechanism of HSV-TK/GCV therapy is shown to be mediated mainly via apoptosis, and necrotic cell death has been shown to play a minor role\textsuperscript{355, 356}. Shortly after
GCV exposure, TAT fusion proteins are released from the GCV-treated dead cells and can diffuse into the neighboring, non-transduced cells, thus enhancing cell killing\textsuperscript{218, 357-359}. The cytotoxic effect is greatly amplified, as the phosphorylated form of GCV is able to diffuse into neighboring unmodified cells primarily through gap junctions. This phenomenon is called the bystander effect. This effect is necessary, as gene-transfer efficiencies of available vectors are inadequate to deliver therapeutic protein into all cells in the tumor, which would be a necessity for complete tumor destruction\textsuperscript{360-363}.

Redirecting Adenoviral Vectors Across the BBB

Given abundant targeting mechanisms, adenovirus looks promising as a platform to design targeted cancer therapeutics supported by numerous preclinical studies. However, few adenovirus-based cancer therapeutics have been proven superior in clinical trials. One of the key hindrances that prevent the successful translation of potent cancer therapeutics from \textit{in vitro} studies to \textit{in vivo} studies stems from their nonoptimal distribution in tumors and inadequate uptake by tumor cells. It has been recognized that the poor and heterogeneous uptake of virus in tumors is a major cause of the failure of oncolytic viral therapy\textsuperscript{331, 364-367}. The inefficient and uneven distribution of therapeutic molecules is most likely due to the much more complicated \textit{in vivo} microenvironment and the three-dimensional structure of solid tumors in contrast to the monolayers of cell culture\textsuperscript{368, 369}. For example, it was found that established solid tumors could be surrounded by basal-membrane-like structures which prevent infiltration of lymphocytes at the tumor sites. Generally, the systemically administrated therapeutic agents (molecules, particles, and cells) reach tumor cells via three steps: distribution through the
vascular compartment, transportation through the microvascular wall, and movement through the interstitium, during which many factors may hamper efficient drug transport, accumulation and retention. These factors include the properties of the drug (size, charge, and configuration) and the physiological condition of the tumor (tissue type, cell density, metabolic environment, blood flow heterogeneities, permeability of the microvascular wall, extracellular matrix components, and physical properties of interstitial fluid).

The physical barriers between tumor and its environment represent a major challenge for cancer therapeutics delivery that make tumor cells inaccessible to systemically administrated drugs\textsuperscript{370}, preventing the effective accumulation of drugs in the tumor sites. For brain tumors, the blood-brain barrier (BBB) and its derived blood-tumor barrier (BTB) represent the major obstacle. A number of methods have been tried to increase the permeability of BBB to facilitate the drug delivery to brain tumors, including chemical or physical means\textsuperscript{370-373}. A typical example is the usage of bradykinin to transiently open BBB/BTB by down-regulating the cell adhesion and cytoskeleton molecules in vascular endothelium, including tight junction-associated proteins zonula occuden-1 (ZO-1), occudin, caludin-5, and filamentous actin\textsuperscript{372}. Even though it was claimed that the permeabilization of vascular endothelium by bradykinin is selective to the BBB of tumor but not to the BBB of normal brain under certain conditions in animal models\textsuperscript{374, 375}, controversy does exist\textsuperscript{376}. Therefore, it should be extremely cautious to apply this procedure to human subjects.

On the other hand, restriction of delivery to precise tumor targets in the brain to avoid adverse effects is also required, which makes gene therapy an obvious choice since it could potentially achieve highly localized and sustained delivery to the tumor.
Therefore, engineering the gene delivery vector so that it can traverse but not damage the BBB, as well as target the tumor, is thus a rational and attractive direction. Since adenovirus re-targeting has been widely explored in terms of specific and effective gene delivery into certain target cells, in the studies we presented in chapter 2, we took advantage of the high efficiency of MTf transcytosis, and applied it in combination with Ad5 vector re-targeting strategy.

Employment of an *in vitro* model is essential to study whether the bi-specific adaptor protein sCAR-MTf was capable of redirecting Ad5 vectors traverse the BBB. In this work, the *in vitro* BBB model was established with BBMVEC cells growing in a monolayer on transwell membranes, which has been utilized elsewhere123, 128, 377-381. The transendothelial electrical resistance (TEER) was monitored to confirm the formation and integrity of a well-sealed endothelial layer before and after the transcytosis assay.

We designed and constructed a bi-specific adaptor protein sCAR-MTf, and examined its ability to redirect Ad5 across the BBB using an *in vitro* BBB model system established with BBMVEC cells. The adaptor protein exhibited specific and strong binding to Ad5 vector, suggesting its capability in vector delivering. The adaptor protein was able to redirect Ad5 vectors to traverse the BBB, often with an efficiency of 40–50 times higher than that of control. The Ad5 viral particles (VPs) that had undergone transcellular transport maintained their functionality/infectivity, as assessed by their gene transfer efficacy. In addition, the adaptor protein mediated Ad5 transcytosis was temperature- and dose dependent, which are the characteristics of receptor-mediated transcytosis, in accordance with previous studies123, 128. Importantly, sCAR-MTf-
mediated Ad5 transcytosis showed strong apical-to-basal preference, supporting its potential utility in transporting Ad5 vectors toward brain tissue.

Currently, no specific receptor has been identified for MTf. Although human MTf shares 39% homology with human serum transferrin\textsuperscript{125}, TfR is found not to be responsible for MTf transcytosis. Instead, a member of LDL receptor family, LDL receptor-related protein (LRP), may play an essential role in this regard\textsuperscript{123}. In fact, LRP may be a common mediator for its binding partners to traverse the BBB. In addition to MTf, LDL, lactoferrin and LDL receptor-associated protein (RAP) have been found to cross the BBB with high efficiency and the receptor involved in their transcellular transport appears to be LRP\textsuperscript{112, 123, 124, 128, 133, 382}. \textit{In vivo} transport across the BBB of these proteins may also help explain the observations that MTf, lactoferrin and LRP accumulate in the brain of patients with neurological diseases such as Alzheimer’s disease\textsuperscript{383-385}. Interestingly, LRP-mediated transcytosis may only be a feature of endothelial cells, as in other cell types or organs, the majority of these proteins, once internalized, are found to be degraded or recycled\textsuperscript{386-388}. The stage of differentiation of endothelial cells also appears to play a role in determining what pathway protein uptake utilizes. For example, in growing brain capillary endothelial cells, LDL is classically internalized by the clathrin pathway, and directed to lysosomes for degradation. However, when the cells are fully differentiated, even though the classic degradation pathway (via lysosomes) is functional, LDL is mostly directed to non-degradation transcytosis pathway\textsuperscript{112}. Our data showed that in the presence of sCAR-MTf, the majority of internalized Ad5 VPs were directed to the transcytosis pathway in the BBB model that is formed by differentiated cells, and very little remained inside the cells. Apparently, the
transcytosis pathway adopts a different trafficking mechanism from the classical endocytosis pathway, as it can bypass the lysosomal degradation. In this regard, caveolae, a type of vesicle that contains enriched caveolin and are non-clathrin coated, has been implicated in LRP-mediated LDL transcytosis through the brain microvascular endothelial cells\textsuperscript{112}. Morphologically, caveolae are spherical invaginations of plasma membrane of regular shape and size (up to 100 nm in diameter), occurring in single or in grape-like clusters. To find out whether the caveolae transcytosis pathway contributes, at least in a part, to the transport of Ad5/adaptor protein complex across the BBB, we sought to explore the role of several caveolae inhibitors in the MTf-mediated Ad5 transcytosis. Filipin, which is a cholesterol scavenging compound, is known to disrupt caveolae, and the thioalkylating agent N-ethylmaleimide (NEM), which interferes with the docking and fusion of vesicles on cell membranes, were commonly used to inhibit caveolae-mediated transcytosis\textsuperscript{389,390}. Regrettably, we observed an increase rather than reduction of MTf-mediated Ad5 transcellular passage after treating the BBMVECs with either filipin (Fig. 1) or NEM. These results may be because bovine brain microvascular endothelial cells in \textit{in vitro} condition respond to filipin and NEM in a very different manner compared to rat lung vascular endothelial cells, in which the inhibiting role of NEM or filipin to caveolae-mediated transcytosis was established\textsuperscript{389,390}. However, it is more likely that those inhibitors are harmful to the BBMVEC in our \textit{in vitro} model and compromise the integrity of the well-sealed monolayer, since the control protein (sCAR) also induced the transcellular passage of Ad5 as high as that induced by MTf protein (Fig. 1). The claim is also supported by the fact that the TEER of the monolayer dramatically decreased after adding filipin (data not shown). The cholesterol-binding agent filipin and
**Figure 1** Effect of filipin on transcytosis in the in vitro BBB model. The endothelial monolayers on transwells was treated with filipin solution (diagonal pattern bars, 0.3, 0.9, 1.5 µg/ml, respectively) for 5 minutes before the transcytosis assay. Ad5 copy numbers in the lower chamber was quantified by real-time PCR. After filipin treatment, the transendothelial transport of Ad5 VPs in both sCAR and sCAR-MTf groups was significantly increased.
the alkylating agent NEM were recently found to increase the vascular permeability\textsuperscript{391,392}. It is not completely understood how filipin and NEM affect microvascular permeability; however, filipin removes sterol components, which are important for proper biological functions of most eukaryotes, and was found to form globular deposits that disrupt the planar organization of cell membranes\textsuperscript{393}. NEM was recently found to inhibit the function of microtubule proteins which are essential to the integrity of all living cells\textsuperscript{394-396}. These facts indicate that filipin or NEM may not be suitable for the specific inhibition of caveolae-mediated transcytosis; and the precise mechanism for MTf-mediated transcytosis of Ad vectors across the BBB, therefore, remains to be investigated.

Although the data presented here were obtained \textit{in vitro}, the proof-of-principle study appears to be very promising. It is noteworthy that the \textit{in vivo} utility of sCAR adaptor protein-mediated Ad5 re-targeting strategy has been directly explored and demonstrated in an earlier study, in which Ad5 vectors were redirected to the lungs expressing carcinoembryonic antigen (CEA) in a mouse model\textsuperscript{199}. Further efforts are rational to focus on investigation of the \textit{in vivo} utility of sCAR-MTf-mediated Ad5 transcytosis, and the means of improving the efficiency of this strategy.

Of course, there are many other factors that influence the biodistribution of viral vectors in tumors after they cross the BBB and reach the tumor parenchyma. For example, differential tumor structure and composition (cell density variance and blood flow heterogeneity) induce severe spatial and temporal heterogeneity of distribution\textsuperscript{369,397}. In the tumor region where blood flow is deficient, the therapeutic molecules pervade mainly by diffusion and convection in interstitial fluid, whose velocity is greatly limited by the fluid pressure and hydraulic conductivity\textsuperscript{398}. The extracellular matrix composition of
solid tumors, which is composed of tumor-associated fibrous proteins (e.g. collagen and elastin) and polysaccharides (e.g. hyaluronan and proteoglycan), also seriously limit the transport of drugs\textsuperscript{398}. Therefore, further studies are demanded for fundamentally improving the drug delivery to brain tumors.

**A Powerful Multifunctional Adenoviral Platform for Cancer Therapy**

The concept of targeting represents the future direction of the development and revolution of cancer therapeutics. An *in vivo* imaging system, which provides a means to study the accumulation, spread, distribution, retention, and antitumor function of cancer therapeutics, could address key issues that are fundamental to the design and test of novel cancer therapies, especially for those replicative virus-based therapeutics\textsuperscript{50, 280, 323, 399}. To combine these functionalities and as a direction to create more potent cancer therapeutics, the NCI promotes the generation of a multi-functional nanoparticle for the detection and treatment of cancer, which are embedded with targeting, imaging and cancer therapeutic modalities (as an example, see the ref.\textsuperscript{53}).

Adenovirus based viral vector has been utilized in a large number of gene therapy studies via a variety of modifications on the capsid proteins, suggesting its potentials in accommodating the aforementioned functional modalities. Recent evidences from our group and others showed that heterologous polypeptide ligands can be genetically incorporated into Ad vector for functional display through the fusion with the C terminus of pIX\textsuperscript{49-52, 251}. Such incorporation showed flexibility in terms of the size and types of the heterologous polypeptides and did not severely compromise Ad’s overall biology and its utility in gene therapy settings. Therefore, we proposed that adenovirus-based vector
system could be a powerful platform to carry all of the three aforementioned functional modalities for the detection and treatment of cancer, i.e. imaging, targeting, and cancer therapeutics, in pIX locales. In chapter 3, we performed a proof-of-principle study to genetically generate a triple pIX mosaic Ad5 vector carrying three different types of pIX molecules in the viral vectors, i.e. IX-Flag, IX-His$_6$, and IX-mRFP1. The native pIX gene of Ad was replaced with three modified pIX genes encoding pIX proteins containing Flag, His$_6$ polypeptides or mRFP1 protein at the C terminus, respectively. The Ad containing double or triple pIX modifications, Ad5-IXFlag-IXmRFP1 and Ad5-IXFlag-IXHis$_6$-IXmRFP1, were successfully rescued.

Our pIX-modified Ads appeared be more thermolabile than control Ad5 as shown in the heat inactivation assay, which is not unexpected since pIX acts as a cement protein stabilizing hexon-hexon interaction and viral particle stability. Of note, Ad5-IXFlag-IXHis$_6$-IXmRFP1 seems to be more thermostable than AdIXmRFP1 and Ad-IXFlag-IXmRFP1. This can be explained by the fact that the capsid of triple pIX modified viruses contains more IX-Flag and IX-His$_6$ proteins, which are small in size and do not affect the cement function of pIX and overall stability of Ad5 virion$^{251}$. In the growth kinetics experiment, the functional multiplicity of control Ad5, Ad5IXmRFP1, Ad5-IXFlag-IXmRFP1, Ad5-IXFlag-IXHis$_6$-IXmRFP1 were 0.050, 0.013, 0.013, 0.020 PFU/cell, respectively. Thus, the 2-log difference of recovered viral progenies after 24 hours between control Ad5 and pIX modified virus could hardly be explained by different initial infection. This, together with the delayed full CPE formation in pIX modified viruses, suggested that modification on pIX had adverse effect on viral replication and/or assembly. These data were consistent with previous studies, in which
deletion, mutation and modification of pIX was suggested to perturb Ad’s stability\textsuperscript{251,255,256,260,261}. In particular, pIX whole protein was essential for genome packaging and stability, and residues 13-15 and 22-28 at the N terminus were vital since deletion of these residues made viruses seriously thermolabile. The central region and C-terminal domains seemed to be trivial in this regard because point mutations (L114P and V117D) and deletions on residues 60-72 (alanine stretch)/100-114 (large part of leucine repeat) neither caused problems in pIX incorporation nor viral thermolability, arguing for a good tolerance of modification in these two regions. However, the properties of heterologous peptide \textit{per se}, such as structure, size and electric charge, may affect the stability\textsuperscript{251}. In addition, pIX has been suggested to be involved in virus-induced nuclear reorganization and the inhibition of cellular antiviral responses\textsuperscript{255,400}, and may act as a transcriptional activator\textsuperscript{401}, although the significance is unclear\textsuperscript{402}. Therefore, structural compromise on leucine repeat at the C terminus and alanine stretch at the central region of pIX may also be a cause of the delayed CPE induction by pIX-modified Ads.

However, such mild compromise in the biology of derived Ad vector caused by the radical pIX modification is considered tolerable for gene therapy application, since the modified viruses was still able to efficiently produce high-titer viral progenies with reasonable VP/PFU ratios.

The three modified pIXs were incorporated into viral capsid as demonstrated by Western blotting analysis on lysed viral protein as well as fluorescence microscopy. The added Flag and His\textsubscript{6} short peptides and the mRFP1 protein in the C termini of pIX molecules was exposed outward on the viral particles as demonstrated by ELISA assay. By labeling pIX modified viruses with three tag-specific antibodies which were
conjugated with gold nanoparticles in three different sizes, we have showed that two or three heterologous IX proteins could coexist on a single virion of Ad5-IXFlag-IXmRFP1 or Ad5-IXFlag-IXHis6-IXmRFP1, respectively. This result provided direct evidence that the triple mosaicism in pIX molecules could be the feature of a single virion rather than mixed viral populations. These results demonstrated our strategy to genetically generate triple pIX mosaic Ad and the possibility to derive multi-functional nano-platform through triple modification of pIX.

Nevertheless, these data also suggested that three types of pIX were not equally incorporated. In Western blotting analysis, IX-Flag protein appeared to be the most and IX-mRFP1 seemed to be the least. Fluorescence microscopy, ELISA and immunogold EM also suggested possible low incorporation rate of IX-mRFP1 protein. The unequivalent stoichiometry of IX proteins in the viral particles could be explained by the uneven expression of three pIX proteins (due to distinct in cis elements being used to drive gene expression, e.g. promoters and enhancers), and/or by the uneven incorporation ability during viral assembly (due to different physical properties of three modified pIX proteins, e.g. size, charge profile, affinity to the viral particle.). In addition, the properties of modified pIX such as size, charge and conformation could also cause aggregation and incompatibility, and interfere with viral assembly (Ugai et al., unpublished data).

The problem of uneven expression of three different pIX proteins could be potentially solved by using a single set of in cis components to drive the co-expression of three different pIX proteins, whose coding sequences are linked by FMDV-2A elements. FMDV-2A elements can mediate “reading skipping” during translation and thereby “cleave” two translational products with even ratio\(^{403, 404}\). It has been shown to drive the
co-expression of up to four genes with high efficiency and achieve almost even “cleavage”\textsuperscript{405}. After “cleavage”, FMDV-2A elements leave their own residues (about 19 amino acids) to translational products, resulting in one amino acid add-on to the N terminus of the 3’ products and 18 amino acids to the C terminus of the 5’ products. These extra sequences are quite small and have not been found to cause any problems to the gene products, and may have beneficial effects such as acting as a tag sequence for easy protein detection\textsuperscript{406}.

We also tried another strategy to produce the triple pIX mosaic virus by co-infecting the helper cell line with three individually pIX-modified adenoviruses, each of which encodes one type of desired pIX proteins. By using this strategy, the even incorporation of three different pIX proteins in the viral particles could be achieved by adjusting the expression of them (simply by adjusting the multiplicity of infection (MOI) of each virus) to complement their affinity discrepancy. This strategy worked well for generating triple mosaic Ad5 carrying both small tag proteins (Flag and His\textsubscript{6}) and a large functional protein, mRFP1 on pIX protein; and their co-existence in a single virion was demonstrated by immuno-gold electron microscopy. However, this strategy may not be favored when applied to clinical investigation since the three parental viruses rather than a single viral vector must be carefully characterized in terms of safety and efficacy, which need a much bigger trial size and much more costs.

It is noteworthy that, although there are 240 copies of pIX molecules in each viral particle, only a few gold nanoparticles were bound on Ad5-IXFlag-IXmRFP1 or Ad5-IXFlag-IXHis\textsubscript{6}-IXmRFP1 virus. This is probably due to spatial hindrance effect of gold particles, and we chose big gold particles for easier differentiation, which have relatively
high spatial hindrance effect against each other during staining. Besides, the low efficiency of triple staining may be the reason of the scattered staining pattern shown in the figure. Therefore, it is not unexpected that the frequency of triply-labeled Ads is low that only about 2% viral particles were positively stained with all three gold particles. Taken together, we have demonstrated for the first time a triple mosaic capsid configuration based on pIX modification, and validated the possibility of further radical engineering in pIX.

We herein presented a genetically modified Ad5 vector carrying three distinct heterologous ligands on its pIX locales, which potentially could serve as a platform to design powerful multifunctional cancer therapeutics for the detection and treatment of brain tumor. It has been demonstrated that imaging and therapeutic ligands/motifs could be incorporated into pIX locales of Ad5 for functional display\textsuperscript{49-52}. With respect to the incorporation of targeting functionality into pIX, previous studies\textsuperscript{267, 407} suggested that transductional targeting via incorporating targeting ligand on the C terminus of pIX usually is not as effective as doing thus on fiber knob, partially due to its relatively low accessibility by virtue of the topology and dimensional arrangement of pIX proteins within GONs and steric hindrance effect from fibers. This issue may be circumvented by lifting the targeting ligand toward the hexon top via alpha-helical spacers to improve accessibility as illustrated by Vellinga \textit{et al.}\textsuperscript{265}, although this hypothesis is under debate\textsuperscript{267, 407}.

Based on the proof-of-principle studies we mentioned above, our triple pIX mosaic vector system seems to be a valuable and reasonable platform to generate multifunctional Ad5 vector carrying targeting, imaging and cancer therapeutic molecules. We
sought to choose a poly lysine peptide (pK) and an anti-CEA single chain antibody (scFv) hMFE, an mRFP1 protein, and an HSV-1 TK protein to exemplify targeting, imaging, and cancer therapeutic functionalities, respectively. Our preliminary data on non-genetically generated triple pIX mosaic Ad5 carrying pK, TK, mRFP1 functional modalities further strengthened our hypothesis (data now shown).

Besides carrying three distinct functionalities for detection and treatment of cancer, the flexibility of our triple pIX mosaic Ad vector platform is useful in the design of viral vectors to fulfill other sophisticated tasks. For example, better understanding of organ and tissue’s physical barriers makes it possible to deliver viral vectors in vivo more efficiently than before by utilizing various transcytosis machineries, e.g. as we described in chapter 2, which needs an extra relaying motif for transcytosis besides the targeting ligand against the receptor of destination. Double imaging ligands with distinct spectral properties on a single viral vector could potentially expand its usage in complicated in vivo environment, e.g. with various detection depth or auto-fluorescence background. A dual imaging system could also significantly reduce the detection background through detecting the co-localization of two imaging signals or using current FRET technology if the two fluorescent proteins are properly chosen.

Double targeting ligands could also be beneficial in the development of targeted cancer therapy. Many other targeted cancer therapies based on antibodies and other small molecules do not perform as remarkable as imatinib does. A major reason of their unsuccessful experience is that the selective nature of these molecules limits their application in subsets of tumors other than the one to which they were initially designed.
to be applied. In other words, the key to the success of targeted molecular medicine relies on the balance between the selectivity (targeting) and effective generalization.

A similar situation exists in obtaining potential benefits to incorporate double therapeutic ligands on a single viral vector. One of the major drawbacks of targeted molecular therapeutics comes from their highly specific, unique anti-tumor machinery, which may result in potent drug resistance especially when the therapeutics is used as a single agent. A direct evidence for this claim is that BCR-ABL positive CML cell lines developed differential sensitivity to STI571 (a potent tyrosine kinase inhibitor) by overexpressing BCR/ABL (the target of STI571), overexpressing P-glycoprotein (Pgp) efflux pumps to reduce the drug uptake, and/or developing compensatory mutations. It is also supported by the phenomenon that most of these drugs exhibit robust anti-tumor effect when combined with other therapies. Therefore, a viral vector incorporated with multi therapeutic molecules with distinct anti-tumor machineries could significantly reduce drug resistance of tumor cells and be of benefit.

In this dissertation, we designed a set of adenoviral vectors with various modifications on capsid proteins, and explored their potential usages in a variety of gene therapy settings, especially in the treatment and detection of brain tumors. The potential of our radically designed adenoviral vectors has been clearly suggested by our in vitro and in vivo experiments; and various questions and problems arisen from our studies also extended our understanding in the development of potent targeted cancer therapeutics in brain tumor as well as other tumor types. Our studies presented herein mainly focused on in vitro or simplified in vivo models for proving concepts, and much work is still needed in terms of combining and optimizing the targeting/re-targeting, imaging, and therapeutic
functionalities within specific tumor scenarios to explore and realize the full utility of our strategies and to translate our work to human subjects.
LIST OF REFERENCES


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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
ANIMAL PROTOCOL APPROVAL
NOTICE OF APPROVAL

DATE:      May 22, 2008
TO:        David T. Curiel, M.D.
            BMRII 502 2180
            FAX: 975-7476
FROM:      Judith A. Kapp, Ph.D., Chair
            Institutional Animal Care and Use Committee
SUBJECT:   Title: Capsid-Labeled Adenovirus for Virotherapy Monitoring
            Sponsor: NIH
            Animal Project Number: 080507516

On April 30, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>A</td>
<td>200</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from May 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 080507516 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.
APPENDIX B

INSTITUTIONAL REVIEW BOARD FOR HUMAN USE
DESIGNATION OF NOT HUMAN SUBJECTS RESEARCH
DATE: 11/14/08

MEMORANDUM

TO: Yizhe Tang
Principal Investigator

FROM: Sheila Moore, CIP
Director, UAB OIRB

RE: Request for Determination—Human Subjects Research
IRB Protocol #N081113003 - Modification of Adenovirus Capsid Proteins for Gene Therapy Applications

An IRB Member has reviewed your application for Designation of Not Human Subjects Research for above referenced proposal.

The reviewer has determined that this proposal is not subject to FDA regulations and is not Human Subjects Research. Note that any changes to the project should be resubmitted to the Office of the IRB for determination.

SM/hw